بسم الله الرحمن الرحيم

PREVALENCE OF AVIAN INFLUENZA VIRUS TYPE A ANTIBODIES IN KHARTOUM STATE AND STUDY OF THREE SERODIAGNOSIS TECHINIQUES

By

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بِسۡمِ ٱللَّهِ ٱلرَّحۡمَٰنِ ٱلرَّحِيمِ

ٱللَّهُ نور ٱلسمـٰوٰتِ و ٱلأرض مثَلُ نورِهِ كَمِشكَوٰةٍ فِيها مِصباح ٱلمِصباح فى زجاجةٍ ٱلزجاجةُ كأنها كَوكَبٌ درىٌ يوقَد مِن شجرة مٰبركَةٍ زيتونةٍ لا شرقيةٍ و لا غَربيةٍ يكاد زيتها يضِىء ولَو لَم تمسسه نارٌ نور علَىٰ نورٍ يهدِى ٱللَّهُ لنورِهِ من يشآءُ و يضرِب ٱللَّهُ ٱلأمثَٰلَ لِلناس و ٱللَّهُ بِكُلّ شىءٍ عَلِيمٌ 35

سورةُ النور

بِسۡمِ ٱللَّهِ ٱلرَّحۡمَٰنِ ٱلرَّحِيمِ

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DEDICATION

To the soul of my father and to my mother,

whom they taught me by example the power of principle.

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I thank Allah as the number of his creatures, complacency of himself, his words ink and his throne weighing, for all blessings gave us. In truth, so many people have contributed so extensively to my learning over the years that it is no longer to say precisely to whom I am indebted.

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ABSTRACT

The objective of the study was to investigate the prevalence of AIV antibody Ab in Khartoum state, and Compare between serological tests; AGID, HI and ELISA. Two hundred and fifty eight blood samples were collected in 2006 from twelve farms in Khartoum state and subjected to serologic screening for type-specific antibodies to type A influenza viruses, there was serologic evidence of past infection with AI virus type A in some flocks. Agar gel immunodiffusion (AGID) and an enzyme-linked immunosorbent assay (ELISA) tests were used for detecting Ab against type A avian influenza (AI) virus. Hemagglutination-inhibition (HI) test was used for subtyping of the corresponding antigen. The sensitivity and specificity of the AI-ELISA was compared in the various groups with those of the AGID and the hemagglutination-inhibition (HI) test; prapered hyperimmune sera and field sera. During the course of preparation of hyperimmune sera (0-80 days) of specific-antibodies-negative (SAN) chickens with AI subtypes A/Mallard/Neth/12100 (H7N3) and Duck/Sing/645.3/97/128 (H5N1), the AGID test was able to detect type A specific Ab as early as 14 days postinoculation(PI). Similarly, the AI-ELISA detected AI Ab and measured rising levels of Ab through 66 days PI, at which time the chickens were re-exposed to AI virus at 21 days post primary inoculation with the same AI subtype. HI detected low levels of AI Ab at 14 days PI. Following a secondary infection, HI measured rising levels of AI-specific Abs (35-80 days PI).

ملخص بالغة العربية للأطروحة

تهدف الدراسة لمعرفة مدى انتشار .لفيروس أنفلونزا الطيور (أ) بولايـة الخرطـوم و اجـراء المقارنـة بـين للاختبـارات السيرولوجية; اختبار الانتشار المزدوج المناعي في الاجار (AGID), منع تلازن الدم (HI) و الاليـزا (ELISA). 258 عينـة دم جمعت في عام 2006 من 12 مزرعة بولاية الخرطوم اخضعت للفحص السيرولوجي للكشف عن للأجـسام المـضادة المعينـة لنوع(أ) من فيروسات الأنفلونزا، وجد دليل للتعرض لعدوى سابقة لنوع (أ) من فيروسات الأنفلونزا في بعض من القطعان. اختبار الانتشار المزدوج المناعي و إلاليزا استعملت للكشف عن الأجسام المضادة ضد النوع (أ) فيـروس أنفلـونزا الطيـور (AI). اختبار منع تلازن الدم أستعمل لتحديد النوع الفرعي من المستضد المطابق. تمت المقارنة بين الاختبارات الثلاث عنـد إجراء الفحص للعينات من المجموعات المختلفة;الامصال التي تحتوى على اجسام مضادة لفيروس انفلونزا الطيور محضرة معملياً و العينات من الحقل. اثناء تحضير الامصال التي تحتوى على اجسام مضادة لفيروس انفلونزا الطيور (0-80 أيام) تم حقن طيور كانت خاليه من وجود الاجسام المضادة المعينـه (SAN) بـــفيروس (أ) أنفلـونزا الطيـور (AI),ذو الأنـواع الفرعيـة A/Mallard/Neth/12100 (H7N3) and Duck/Sing/645.3/97/128 (H5N1), وجـد ان اختبـار الانتـشار المزدوج المناعي في الاجار كان قادراً على اكتشاف الأجسام المضادة ألمحدده للنوع لفيروس خـلال 14 يـوم بعـد حقـن الفيروس. كذلك اختبار إلاليزا (ELISA) كان قادر على اكتشاف الأجسام المضادة ألمحدده ، بالإضافة لقيـاس مـستويات ارتفاع الأجسام المضادة خلال 66 يوم بعد حقن الفيروس ، في ذلك الوقت تم إعطاء الطيور جرعـة أضـافية يـوم 21 بعـد الجرعة الأولى بنفس الأنواع الفرعية من الفيروس. اختبار منع تلازن الدم بـداء باكتـشاف المـستويات المنخفـضة للأجـسام المضادة عند اليوم 14 بعد حقن الفيروس و قياس ارتفاع الأجسام المضادة بعد االجرعة الاضافية خلال الأيـام (35-80) مـن بداية التجربة.

INTRODUCTION

Avian influenza (AI) is an OIE notifiable disease, which in its highly pathogenic avian influenza (HPAI) form has become a disease of great importance for animal health and with serious potential threat for human health. HPAI is an extremely contagious viral disease that can affect all species of birds (OIE, 2004). The OIE Terrestrial Code defined notifiable Avian Influenza (NAI) as: "an infection of poultry caused by any influenza A virus of the H5 or H7 subtypes or by any AI virus with an intravenous pathogenicity index (IVPI) greater than 1.2 as an alternative at least 75% mortality" (OIE, 2005).

Antigenic variation is one of the most striking features of the influenza virus; that associated with major antigenic changes in the surface antigens of the virus; occur at irregular and infrequent intervals. Antigenic variation cause epidemiological problems, which are unique in terms of pandemic nature of the disease, its unpredictability and the constantly changing antigenic character of the infectious agent (Schild and Dowdle, 1975). The vaccination is the crucial point in handling the spread of avian flu, but it consider as weapon with two boundaries, because it would hinder the serological survey to monitor virus spread (Beard, 1987), more over; the possibility also exist that vaccine may contribute to maintenance of viruses and continuation of the disease problem by the generation of a virulent antigenic variants (Wood *et al.,* 1985; and Hinshaw *et al.,* 1990).

The increased impact of AI in animal and human health has highlighted the need for more scientific investigation on several aspects of the disease, which has hampered the adequate management of the recent epidemic crises resulting in millions of dead birds and raised concern over loss of human lives and over management of the pandemic potential. The period between introduction of the virus and notification is indicated as the high-risk period (HRP). During the

Dutch outbreak, in the HRP the virus could almost spread freely between flocks, since insufficient biosecurity measures were in place (Stegeman *et al.,* 2004). The HRP can be reduced by implementing syndrome surveillance programmes compelling poultry farmers to report disease problems to their veterinary health authority. In case syndrome surveillance programmes are not implemented, serological monitoring program could be used to detect subclinical low pathogenic avian influenza viruses (LPAI) infections or executed as a complementary measure to syndrome surveillance programs. A second aim of serological monitoring systems is to prove that a certain region is free of disease (EFSA, 2005).

It is well established that lower animals and birds form a reservoir of several different antigenic types of influenza A viruses (Schild and Dowdle, 1975). It is important to understand the epidemiological behavior of the influenza, which can be achieved by extensive and continued surveillance; include, the identification and the antigenic characterization of the influenza viruses that currently prevalent; and serological surveillance to reveal the degree of exposure to past and current influenza variants and the probable level of immunity, particularly to newly emerged variants (Schild and Dowdle, 1975). In addition, it become apparent that HPAI viruses can mutate from LPAI (Garcia *et al.,* 1996, Perdue *et al.,* 1998), even the low pathogenic form of the virus, it is necessary to be monitored and controlled. The HPAI in the intensive layer industry in Pennsylvania in 1983-1984 was the first outbreak which required the culling of about 17 000000 birds to control it. The outbreak resulted particularly difficult to extinguish due to the extensive circulation of the LPAI virus prior to mutation to HPAI, which hampered and delayed diagnostic interventions (Utterback, 1984).

Outbreaks of HPAI subtype H5N1 in some commercial poultry in Khartoum and Algazera states (Ali and Kheir, 2007), not only caused tremendous losses, but also threaten the growing poultry industry sector in Sudan. So the objectives of this study were:

1) Study the prevalence of AIV antibody Ab in Khartoum state.
2) Compare serological tests; AGID and HI test using local standardized reagents, ELISA (commercial kit), which are used in serological surveillance.

Chapter 1

LITERATURE REVIEW

1.1 Background of influenza disease

Influenza is a disease of economic importance caused by a zoonotic virus that occurs in lower animals and bird as well as in humans. Influenza viruses belong to the Orthomyxoviridae Family which constitutes enveloped RNA virions (Webster *et al.,* 2002). The family contains five Genera; influenza virus A, B, C, Thogotovirus, and Isavirus. The term "Avian influenza-AI" is used to describe influenza A subtypes that primarily affect chickens, turkeys, guinea fowls, migratory waterfowl, and other avian species. AI is an ecological classification that does not correspond exactly to other classification schemes (CIDRAP, 2007). The disease signs associated with influenza A viruses (IAVs) infections in avian species vary considerably. Infections caused by most strains of IAVs are completely a symptomatic; however, a few strains -a few members of the H5 and H7 subtypes- produce systemic infection accompanied by central nervous system involvement, with high mortality (Webster *et al*., 1992). In human, influenza virus replicates in straight forward fashion as productive infection without apparent recourse to latency, neoplastic induction or other virus-host relationship, to produce an acute inflammatory disease focused in the trachea that attended by severe but briefly sustained fever and prostration (Kilbourne, 1975).

Influenza, an unvarying disease caused by a varying virus, that unique among infectious agents in its antigenic variability. Coincident with the growing evidence of the epidemiology complexity of

influenza, its virus has proved increasingly interesting as an object of laboratory study- the first virus for which hemagglutination (HA) and hemagglutination inhibition (HI) was demonstrated (Hirst, 1941), the animal virus first show to undergo "recombination" (Burnet and Lind, 1949), the virus first demonstrated to contain an enzyme as a structural protein (Laver and Kilbourne, 1966). And the virus first showed to reproduce by budding from the plasma membrane of the host cell (Holland and Keihn, 1970).

Influenza is the paradigm of a viral disease; in spite of restriction of host range due to Polymerase (PB2), neuraminidase (NA), hemagglutinin (HA) and nucleoprotein (NP) genes. The virus is in continuing evolutions which have paramount importance for epidemics and pandemic of the disease. Understanding of the molecular mechanism of such genes and how restriction is breached would provide knowledge important for preventing future influenza pandemic (Vines *et al.*, 1998). The gene pool of IAVs in aquatic birds provides all the genetic diversity required for the emergence of pandemic IAVs for humans, lower animals and birds (Webster *et al.*, 1992).

1.2 Disease's history

Fowl plaque has been known since 1880, when it has recognized in Italy. Early in last century it spread throughout the great part of Europe. In 1923 the virus was brought to the United States illegally by a laboratory worker and then spread throughout the country (Hagan and Bruner, 1961). IAVs have been isolated at the beginning of the 20th century from chickens, from pigs in the 1920, from human in the 1930, from horses and domestic ducks in the 1950, from terns in 1960 and from many waterfowl and shorebirds since 1979 (Kowaoka *et al.*, 1988; Webster *et al.*, 1992).

Several pandemics emerged during last century leading to drastic losses in human. Of these, the 1918-1919 "Spanish flu" pandemic was among the deadliest public health crises in human history, killing an estimated 50-100 million people world wide (Johnson and Mueller, 2002; Morens and fauci, 2007). The 1957 (H2N2) and 1968 (H3N2) pandemics, were resulted from reassortment between circulating descendants of the 1918 human virus and circulating AI strains, while the 1918 (H1N1) pandemic apparently arose by genetic adaptation of an existing AI virus to human (Scholtissek *et al.*, 1978; Taubenberger and Morens, 2006; Morens and Fauci, 2007). In 1996 highly pathogenic H5N1 was isolated from a farmed goose in China (WHO, 2007). In the fall of 2003 and summer 2004 an outbreak of highly pathogenic avian influenza (HPAI) caused by H5N1 started in Asia, and spread in domestic poultry farms at a historically unprecedented rate. In the summer of 2005, H5N1 began expanding its geographic range beyond Asia; this trend has continued into 2007 (CIDRAP, 2007). Recent H5N1 is responsible for the current epizootics among birds in Asia, Europe, and Africa, have been associated with sporadic human fatalities, concern has been raised that new pandemic, as fatal as the pandemic of 1918, or more so, could be developing (Morens and Fauci, 2007). Since 1959 only 24 primary isolates of HPAI viruses from domestic poultry have been reported, but six of these having the greatest socio-economic impact have occurred in the last eight years e.g. outbreaks in 1999/2000 in Italy, 2003 in The Netherlands. Recently the USA and Canada have also experienced HPAI disease outbreaks (EFSA, 2005).

1.3 Avian influenza in Sudan

Elmubarak, (1970) stated that Fowl plaque was reported in Sudan since 1925, but the virus did not appear to have been isolated. In February 1998 an outbreak of the disease occurred in a farm in

Khartoum North area and the virus of influenza type A was isolated, and antibodies (Abs) against AIV were detected during serological survey (Elamin, 2000). In 2006 H5N1 was isolated from an outbreak that occurred in Khartoum and Algazeera states (Ali and Kheir, 2007). The disease caused heavy economical losses during 2006 outbreaks, as a result of depopulation of chicken flocks and the subsequent shortage in poultry products.

1.4 Etiology of avian influenza

Two groups of AI viruses have been isolated from birds. The first group of viruses includes the H5 and H7 subtypes, have virulent variants that are able to replicate in most organs, producing systemic HPAI and may cause 100% mortality in domestic poultry (Webster *et al.,* 1992; Garcia *et al*., 1998). These viruses were known to be the causative agent of fowl plague, and later, they were identified by Schafer (1955) as influenza viruses. The other group is less virulent; low pathogenic avian influenza (LPAI) includes the majority of field isolate that have a variety of HA subtypes (Webster *et al.,* 1992), that replicate only in the respiratory and intestinal organs and cause no or low mortality (Garcia *et al*., 1998), but these viruses can serve as progenitors to HPAI viruses (Garcia *et al*., 1996; Perdue *et al*., 1998). The major determinant of the difference in virulence between the two groups of viruses is susceptibility of the HA0 to the host proteases which requires for post translational cleavage into HA1 and HA2 (Webster and Rott, 1987; Webster *et al.,* 1992; Rott, 1992). The HA0 precursor proteins of LPAI viruses have a single arginine at the cleavage site and another at position -3 or -4. These viruses are limited to cleavage only by certain host proteases such as trypsin-like enzymes and are thus restricted to replication at sites in the host where such enzymes are found, i.e. the respiratory and intestinal tracts. The HA0 proteins of HPAI viruses

possess multiple basic amino acids [arginine and lysine] at their HA0 cleavage sites either as a result of apparent insertion or apparent substitution and appear to be cleavable by ubiquitous proteases (Vey *et al.*, 1992; Wood *et al.*, 1993; Senne *et al.*, 1996).

Genetic characterization of H5N1 strains involved in a recent epizootic has demonstrated two distinct phylogenetic clades (Webster *et al.*, 2006). Clade 1 viruses circulated in Cambodia, Thailand, and Vietnam, and clade 2 viruses circulated in China and Indonesia and spread westward to the Middle East, Europe, and Africa. Six different subclades of clade 2 have been recognized; three of these are responsible for recent human H5N1 cases (Chutinimitkul *et al.*, 2007).

1.5 Classification of influenza virus

Orthomyxoviridae is RNA Family constitutes enveloped virions with 80-120 nm in diameter and 200 to 300 nm long. The family contains five Genera; influenza virus *A, B, C*, Thogotovirus, and Isavirus, classified by variation in NP and M antigens (Webster *et al.*, 2002; and CIDRAP, 2007). As Orthomyxoviruses, the influenza viruses bear certain striking similarities to the larger parainfluenza viruses, including the possession of negative stranded RNA and a virion transcriptase, external envelope that contains glycoprotein spikes (HA and NA) and respiratory tract pathogenicity. Influenza viruses exist as three biologically similar, but antigenically heterologous types A, B and C. These viruses share no virus-coded antigens in common, differ in epidemiology and probably to some degree in the severity of illness that they cause. Genetic recombination, or complementation, among these types has not been verified but the IAVs possess a segmented genome that undergoes high frequency of genetic recombination or reassortment (Kilbourne, 1975).

The identification of IAVs as the type based on two stable, type-specific internal antigens: NP and M, and as subtype is based on the strain-specific surface antigens: (HA) and (NA) (Dowdle *et al.*, 1974). HA and NA is highly mutable, nine types of NA (N1, to N9), and until recently, 15 HA types had been recognized, but a new type (H16) was isolated from black-headed gulls caught in Sweden and the Netherlands in 1999 and reported in the literature in 2005 (Fouchier *et al.*, 2005); they are not serologically cross reactive. Different variants of several subtypes are known (Webster *et al.*, 1992). Subtypes of IAVs may include several variants which are partially serologically cross-reactive; only one such subtype is present in the human population at any one time. In case of animal influenza viruses, antigenically different subtypes can coexist (Kilbourne, 1975).

1.6 Virion properties

1.6.1 Morphology

The Influenza virion is roughly spherical, contains host-derived lipid bilayer envelope in which virus-encoded glycoprotein spikes (HA and NA), Matrix (M1), and inner shell matrix (M2) are embedded; at the center, NP contain segmented RNA (Kilbourne, 1975; Webster *et al.*, 1992).

1.6.2 Chemical composition.

1.6.2.1 Proteins

Ten Polypeptides are coded by the viral genome; represent the largest portion of the virion. These include three transcriptases (PB1, PB2, and PA), two surface glycoproteins (HA, and NA), two

matrix proteins (M1 and M2), one NP, and two nonstructural protein (NS1 and NS2) (Zebedee and Lamb, 1988: Webster *et al.*, 1992) their molecular weight range from 83,500 to 26,500 Daltons, with the total molecular weight of 380,500 Dalton, the two major proteins are NP and M1 (Compans e*t al.,* 1970). The viral proteins could be identified immunologically, i.e. NP, M1, HA, and NA (Choppin and compans*,* 1975). Although the influenza virion is assemble by budding from the plasma membrane of the host cell, it lakes host protein (Holland and Keihn, 1970).

Four of these polypeptides appear to be covalently linked with carbohydrate, i.e. glycoprotein, associated with the surface protein components which are HA (HA1 and HA2), NA, and HA precursor (Rhodes and Van Rooyen, 1968; and Compans e*t al.,* 1970).

1.6.2.2 Ribonucleic Acid (RNA)

The essential point regarding influenza virus RNA, is its segmented nature. RNA segments vary in molecular weight from 1.05×10^6 to 3.5×10^5 (Bishop *et al., 1971*). The single-stranded RNA molecules are of non message (negative) i.e. complementary to the mRNA polarity; makeup between 0.7 to 1% of total particle mass (Rhodes and Van Rooyen, 1968). The base composition consists of guanine, 20.7%; adenine, 26.4%; cytosine 22.4%, and Uracil, 30.5% (Sokol and Schramek, 1962). The base composition of viral RNA varied slightly but significantly from strain to strain (Ada and Perry, 1956).

1.6.2.3 Carbohydrates

Carbohydrates represent 5-8% of total viron mass (Formmhagen e*t al.,* 1959). Most if not all carbohydrates are bound covalently to glycoprotein or glycolipid (Choppin and compans*,* 1975), which are synthesized after infection (Compans *et al*., 1970; and Schulze, 1970). The carbohydrate represents host cell-specific antigen has been found in purified virion (Laver and Webster, 1966).

1.6.2.4 Lipids

A significant portion of virion is lipid, 18 to 37 %, composed of cholesterol, phosphates, and traces of neutral fat in addition small amount of fatty acid and traces of triglycerides have been found in one strain of influenza A. The lipid component is derived from components present in the host cell before infection, although an appreciable amount of the phosphate appears to be synthesized after infection (Kates *et al.,* 1961).

1.6.3 Biological properties

Influenza viruses exhibit some properties that are not shared by other viruses having single-stranded RNA, e.g. genetic recombination and Von Magnus phenomenon (Scholtissek *et al.,* 1966).

1.6.3.1 Genetic recombination and multiplicity reactivation

As the result of segmented nature of RNA, genetic interchange could occur between two different subtypes of IAVs, when cell is doubly infected with them, random incorporation of segments generates progeny viruses containing novel combinations of genes; NA of one parent and HA and internal protein of the other (Laver and Kilbourne, 1966; and Webster *et al.,* 1992).

1.6.3.2 Von Magnus phenomenon

Von Magnus described the production of noninfectious influenza virus as a result of multiple infection of chorioallontoic of embryonated chicken's eggs cells (Von Magnus, 1951). Viruses which occur after serial undiluted passage of the viruses are incomplete in a genetic sense, having lost some of their RNA segment during these passages (Scholtissek *et al.,* 1966). These virus particles have a surface structure very similar to infectious virus, they are immunogenic but they contain less RNA and NP antigen, show a lower infectivity to hemagglutinin ratio and contain more lipid than complete virus particles (Scholtissek and Klenk, 1975).

Some explanations exist for the mechanism of formation of such virus particles: either all virus RNA molecules are synthesized in the cell but, because of defective maturation, are not incorporated into infectious virions, or there is a defective synthesis of virus RNA's inside the cell and progeny virus particles contain a defective virus genome (Nayak, 1972). Meter-Ewert and Dimmock demonstrated that the Von Magnus phenomenon depends on the balance between viral neuraminidase and cell substrate (Meter-Ewert and Dimmock, 1970).

1.6.3.3 Hemagglutination

Influenza virus agglutinates fowl and some mammalian red blood cells (RBCs), due to presence of specific sialic acid containing receptors on their membrane (Winzler, 1969), and HA on virus surface which are responsible for the hemagglutinating activity. When HAs are removed both infectivity and hemagglutinating activity are lost. Sialic acid residues occupy terminal positions on the carbohydrate chains and can be eluted from the receptor by viral neuraminidase (Schulze,

1975). Hemagglutination has proven to be a valuable technique for virus identification, quantitation and purification (Hirst, 1941). Agglutination of RBCs from different animal species used to assess the receptor specificity of IAVs, i.e. human viruses agglutinated erythrocytes from chickens, ducks, guinea pigs and sheep but not horses or cow; while, avian and equine viruses agglutinated all of those erythrocytes (Ito *et al.,* 1997).

1.7 Ultrastructure of AI viruses

The influenza virion is roughly spherical and contains three distinct surface antigens; two of which are glycoprotein spikes; the HA -radial projections- predominates and the NA occurs in patches, approximately 16 nm long. The third is a host cell antigen (Harboe, 1963; Laver and Webster, 1966; Laver and Valentine, 1968; and Webster *et al.,* 1992). Inside the lipid bilayer are eight segments of single-stranded RNA. The RNA is loosely encapsidated by multiple NP molecules. The NP-RNA-polymerase complex contain three viral polymerase proteins (PB1, PB2, and PA), which are situated at the end of the NP (Pons, 1976; Palese and Schulman, 1976; and Webster *et al.,* 1992).

1.8 Physical characteristics of viruses

Viruses remain infectious after 24 to 48 hours on nonporous environmental surfaces and less than 12 hours on porous surfaces (*Bean et al.,* 1982). AI viruses are more stable at low pH than human strains and retain infectivity for over 30 days in non-chlorinated river water at 0°C and for 4 days at 22°C (Webster *et al.,* 1978), and persist in distilled water for 207 days at 17°C and 102 days at 28°C (Stallknecht *et al.,* 1990). The virus survives for years at -70°C or after lyophilization. Infective

tissue retains activity of the virus for months in 50% glycerol-saline at 0°C. Infectivity of human strains in aerosol decays more rapidly than that of avian strains, maximum stability between pH 7 and 8 (Andrewes and Pereira, 1972). Inactivation of virus occurs under following conditions: 30 minutes at 56°C, exposure to oxidizing agents such as sodium dodecyl-sulfate, lipid solvents, and B-propiolactone; and disinfectants such as formalin and iodine (Andrewes and Pereira, 1972).

1.9 Viral replication

Influenza virus replication cycle has several stages which are;

1.9.1 Adsorption

Infection of a cell by a virus is initiated by adsorption, which requires two complementary structures, the glycoprotein or glycolipid receptor site on the cell host surface, that contain terminal sialic acid, and the viral component responsible for recognition of the receptor sites, which is cleavage-activated HA (Sholtissek and Klenk, 1975; Webster *et al.,* 1992).

1.9.2 Penetration and uncoating

Two different mechanisms have been proposed for penetration and uncoating;

1.9.2.1 Viropexis-engulfment

It is an endocytosis process whereby whole virus particles are incorporated into endocytosed vesicle that subsequently fuses with lysosomes (Fazekas and Hall, 1948). The low pH of the

endocytotic vesicle triggers a conformational change in the cleaved HA which is believed to facilitate insertion of the hydrophobic free amino terminus of HA2 into the vesicular membrane, initiating fusion (Webster *et al.,* 1992).

1.9.2.2 Fusion with host cell membrane

Penetration may result from fusion of the viral envelope with the host cell membrane, then releases the virion's content into the cytoplasm of the cell (Morgan and Rose, 1968; Yoshimura *et al.,* 1982).

1.9.3 Transcription

After uncoating, the single stranded virion RNA has to be transcribed into complementary RNA (cRNA). The nucleocapsids of the parent virus migrate into the host cell nucleus, and their associated polymerase complexes begin primary the transcription of messenger RNA (mRNA). Translation of host mRNA is blocked. The Primary transcripts are used for translation of viral proteins NP and NS1, which in the early stage of infection are predominant. Newly synthesized NP and NS1 migrate to the nucleus. The increase concentration of free NP triggers the shift from mRNA synthesis to the cRNA and viral RNA (vRNA) synthesis by infecting viral genome. Newly synthesized vRNA are encapsidated in NP within the nucleus and function as template for secondary transcription of viral mRNAs. Later in infection, the principal translation products are M1, HA and NA proteins. M1 build up in the nucleus is associated with migration of nucleocapsids out of the nucleus for assembly into progeny viral particles in the cytoplasm. HA and NA proteins are posttranslationally processed and transported to the cell surface, where they integrated into the cell membrane (Webster *et al.,* 1992).

1.9.4 Assembling and budding

A viral core of nucleocapsids becomes encased in a shell of M1 protein and buds outward through the cell membrane, enclosing itself within a bubble of membrane as its own envelope, complete with viral surface glycoproteins. Interactions between M1 and the Cytoplasmic dominants of HA, NA, or M2 have been proposed as signals for budding. NA activity of progeny virions releases them from the host cell (Webster *et al.,* 1992).

1.9.5 Maturation

The final step in the virus maturation is extra cellular; this is the cleavage of HA0 (uncleaved HA) into HA1 and HA2. This cleavage is accomplished by host-produced trypsin like proteases. Cleaved HA is relatively unstable at low pH; therefore, with AI viruses, which are transmitted primarily by the fecal, oral route, cleavage probably occurs after excreted virion have entered into their new host and passed through the stomach, HAs cleaved intracellular. HAs of mammalian influenza viruses are probably cleaved by extra cellular proteases of the respiratory tract in either the original or the new host (Webster *et al.,* 1992).

1.10 Epidemiology of influenza

1.10.1 Host range

IAVs infect human, a variety of animals; various bird species; include Wild bird and domestic poultry (turkeys and chickens), pigs, horses, and sea mammals (Andrewes and Pereira 1972). There

is convincing evidence that all 16 HAs subtypes of IAVs are perpetuated in the aquatic bird, especially in ducks, shorebird and gulls. The avirulent nature of IAVs infection in ducks may be as the result of virus adaptation to this host over many centuries, creating a reservoir that ensures perpetuation of the virus. This suggests that ducks occupy a unique and very important position in the natural history of influenza viruses (Webster *et al.,* 1992). Studies on the ecology of influenza viruses have led to the hypothesis that all IAVs derive from the AI reservoir. Phylogenetic analyses of the nucleoprotein gene show that AI viruses have evolved into five host-specific lineages: a classical equine lineage, which has not been isolated in over 15 years; a recent equine lineage; a lineage in gulls; one in swine; and one in human (Webster *et al.,* 1992; and Webster *et al*., 2002).

Certain subtypes of IAVs predominate in wild ducks in a particular flyway to another and from year to year (Hinshaw *et al*., 1985). Starling and sparrows are susceptible to infection and potential spreaders of viruses (Nestorowicz *et al,*. 1987; Webster *et al.,* 1992). Influenza viruses were isolated form pet birds. Since the prevalence of influenza viruses in these birds is limited, their role in evolution and maintenance of the viruses is unknown (Nerome *et al.,* 1978). H5N1 experimentally shown to infect cats (Rimmelzwaan *et al*, 2006); pigeons were found to be resistant or minimally susceptible to infection with HPAI viruses or LPAI viruses (Webster *et al*., 2002).

1.10.2 Transmission and spread

HPAI is some what self-limiting since the high mortality leaves few birds to serve as carriers (Hagan and Bruner, 1961). Transmission could be by ingestion, inhalation, when infected birds shed the virus in oculonasal discharges and faeces (AVMS, 2006), and some believe that insect

vectors may play a role, since there is a viremia. (Beard, 1992) showed the possibility of vertical transmission by isolation of the virus from the albumen and yolk of eggs laid by infected hens. Waterfowl typically experience asymptomatic infection and transmit influenza viruses by the fecal-oral route through contaminated water (Webster *et al.,* 1992), exerting little selection pressure on viral evolution. To jump to new host such as chicken or mammals and infect very different cell types, such as human lung cell, rather than duck enteric (Morens and Fauci, 2007). The susceptibility of duck to infection with IAVs and possibility of transmission to other animal species through the water supply suggests that ducks may be important in ecology of IAVs (Webster *et al.,* 1978).

A peculiar condition is that the influenza usually does not readily pass from one species to another by ordinary contacts (Hagan and Bruner, 1961). Although AI viruses may be transmitted from animals to humans in two main ways; either directly from bird or from avian virus contaminated environment to people, or through an intermediate host, such as a pig (CDC, 2005).

1.10.3 Geographical distribution

In the north hemisphere, IAVs predominate in August and September. Juvenile birds are infected, up to 20%, prior to migration; causing annual epidemic influenza in wild ducks of with no disease signs. During southern migration from Canada, the birds continue to shed virus as well as they come back. IAVs are perpetuated in a particular avian species in the United States (Webster *et al.,* 1992).

In Japan IAVs viruses were isolated during winter months from whistling Swans (Otsuki *et al.,* 1987). IAVs with a variety of HA and NA subtypes have been isolated from various wild bird species in China, Western Europe, Israel, Australia. Africa, particular its Sub-Saharan region, constitutes a seasonal shelter for a large number of Eurasian water birds, provides opportunities for transmission of IAVs between different populations and spread of IAVs over extensive area in both Eurasia and Africa, many IAV subtypes were isolated from them (Gaidet *et al.,* 2007). Some studies demonstrate that evolution of influenza viruses can be influenced by the physical barriers that prevent intermixing of their hosts (Donis *et al.,* 1989; and Gorman *et al.,* 1990).

1.10.4 Antigencity of the influenza Virus

RNA viruses have been recognized as highly mutable since the early studies, owing to the shorter generation times for replicating RNA viruses and the error-prone nature; lack of proofreading among RNA polymerase and repair mechanism. The extraordinary evolutionary success of RNA viruses is attributable to their ability to use different replication strategies and adapt themselves to the widely varying biological niches encountered during virus spread in a single host or multi-host network (Steinhauer and Holland, 1987). Beside that, the segmented nature of the influenza virus genome accounts for recombination in vivo between two different influenza type A viruses (Webster *et al.,* 1972). Recombination can occur between influenza viruses from man and lower animals which offer an explanation for the origin of new strains of pandemic influenza viruses. Recombinant viruses are formed with high frequency; up to 97% frequencies have been obtained (Webster and Laver 1975).

Continuing evolution is most prominent in the surface glycoprotein of influenza viruses but also occurs in each of the eight gene segments of both type A and type B influenza viruses (Webster *et al.,* 1992). The variability results from accumulation of molecular changes in the eight RNA segments that can occur by a number of different mechanisms including: point mutations (antigenic drift), gene reassortment (genetic shift), defective-interfering particles, and RNA recombination (Scholtissek *et al.,* 1978). Each of these mechanisms may contribute to the evolution of influenza viruses to be infectious (Steinhauer and Holland, 1987; and Webster *et al.,* 1992). In humans, pigs, and horses, IAVs show both antigenic drift and shift. In contrast, there is emerging evidence that AI viruses are in evolutionary stasis (Webster *et al.,* 1992). Genetic interaction can occur between all IAVs of man, lower mammals, and that of avian species of influenza viruses (Webster *et al.,* 1972).

1.10.5 Clinical signs

Disease signs vary from mild respiratory involvement to rapidly fatal disease; depending on the species and the age of bird, strain of virus and accompanying bacterial infection. Typical signs of HPAI in chickens or turkeys include; decreased egg production; respiratory signs; rales; excessive lacrimation; sinusitis; cyanosis of unfeathered skin, especially of the combs and wattles; edema of the head and face; ruffled feather; diarrhea; and nervous disorder (Hagan and Bruner, 1961; Webster *et al.,* 1992; and Alexander, 2002). Duck influenza is often associated with sinusitis (Andrewes and Pereira, 1972). Severe disease generally does not develop in wild birds; however, recently H5N1 has been shown to be virulent for wild bird species. An outbreak of H5N1 among

migratory geese, wild swans and other wild birds were occurred (Chen *et al.,* 2005; Olsen *et al.,* 2006).

1.10.6 Pathogenesis

Inhalation of virus into the lung with deposition on the wall of a bronchiole, then the virus adsorbs to the surface of a respiratory epithelial cell by sialic acid receptor. Viral mucinase ridding it from respiratory mucus; then the virus replicates inside cell and liberates to the respiratory surface, spread to adjacent cells mainly in the direction of cilliary beat toward larynx; the spread to the other sectors of bronchial tree, possibly by the inhalation after rales (Burnet, 1960). HPAI viruses have capability to spread beyond the respiratory tract, replicate, produce lesions, degeneration, and necrosis and result in an inflammation (Mo *et al.,* 1997).

1.10.7 Intravenous pathogenicity index (IVPI) test

The virulence of an isolate for domestic poultry should be assessed, by injection of $^{1}/_{10}$ diluted infective allantoic fluid intravenously into each of ten 6-weeks-old SPF (Specific pathogen free) chickens. Birds are then observed for ten days, the isolate is considered to be highly pathogenic; if they cause more than 75% mortality within 8 days (Webster *et al.,* 2002). The OIE criteria for classifying an AI virus as HPAI is: "Any influenza virus that is lethal for six, seven or eight of eight 4 – to 8-week-old susceptible chickens within 10 days following intravenous inoculation with 0.2 ml of a $^{1}/_{10}$ dilution of a bacteria-free, infective allantoic fluid".

1.10.8 Excretion of virus

HPAI viruses present in the blood during all stages of the disease, in all organs and tissues and in all secretion and excretion. The concentration in the blood is great since as little as 0.000,001 ml will regularly infect by paranteral inoculation (Hagan and Bruner, 1961). In aquatic birds, influenza viruses replicate preferentially in the cells lining of the intestinal tract and are excreted in high concentrations in the faeces, up to $10^{8.7}$ 50% egg infectious doses per gram (Webster *et al.,* 1978).

1.10.9 Postmortem finding

Postmortem lesions vary according to virus strain and host. In severe infections there is multiple focal necrosis in most organs, some strains are particularly associated with pancreatic necrosis. Birds that die peracutely may show minimal gross lesions, consisting of dehydration and congestion of viscera and muscles (Andrewes and Pereira, 1972). In birds that die after a prolonged clinical course, petechial and ecchymotic hemorrhages occur throughout the body, particularly in the larynx, trachea, proventriculus and epicardial fat, and on serosal surfaces adjacent to the sternum. There is extensive subcutaneous edema, particularly around the head and hocks. The carcass may be dehydrated. Yellow or grey necrotic foci may be present in the spleen, liver, kidneys and lungs. The air sac may contain exudates. The spleen may be enlarged and hemorrhagic (EC, 2006).

AI is characterized histologically by vascular disturbances leading to edema, hemorrhages and perivascular cuffing, especially in the myocardium, spleen, lungs, brain, pancreas and wattles. Necrotic foci are present in the lungs, liver and kidneys. The nervous system appears normal, but

microscopically shows diffuse encephalitis, degeneration of nerve, and necrotic foci, around which there is proliferation of glia cell (Gliosis) (Hagan and Bruner, 1961).

1.11 Diagnosis

1.11.1 Clinical diagnosis

Variation of signs makes clinical diagnosis difficult, HPAI is characterized by short incubation period of 3 to 5 days; symptom may show within 24-36 hours. A high temperature rapidly develops (110 to 112F), commonly falls to subnormal shortly before death, the appetite is lost and the birds rapidly become lethargic, cessation of production in layer flock. The comb and wattle commonly become bluish black. A mucoid nasal discharge appears, and often edema of head and neck develops. The course of the disease is very rapid, deaths usually occur within a few hours after the appearance of the symptoms. The mortality rate is high, may reach 100% (Hagan and Bruner, 1961). Postmortem lesions also vary according to strains, HPAI viruses produce lesion in respiratory and enteric tissue while LPAI viruses produce lesion only in respiratory tissue (Mo *et al.,* 1997).

1.11.2 Differential diagnosis

In the differential diagnosis of HPAI, the following diseases, in particular, must be considered: (I) other diseases causing sudden high mortality, such as: Newcastle disease; infectious laryngotracheitis; duck plague; acute poisonings (EC, 2006).

(II) other diseases causing swelling of the combs and wattles, such as: acute fowl cholera and other septicaemic diseases; bacterial cellulitis of the comb and wattles (EC, 2006).

1.11.3 Laboratory diagnosis

1.11.3.1 Collection of specimens

1.11.3.1.1 Specimens for agent detection

In mammals including humans, pigs and horses influenza is primary a respiratory tract infection, while in avian species influenza can be an infection of both the respiratory and large intestinal tract (Webster *et al.,* 2002). So appropriate samples for agent detection from live bird are swabs from trachea and cloaca or fresh faecal samples, should be taken during the first 3 days of infection. Intestinal contents or cloacal swabs and oro-nasal swabs, plus brain, spleen, heart, lung, pancreas, liver and kidney should be taken from dead bird and processed either separately or as a pool (Alexander, 2000; and Webster *et al.,* 2002). Samples should be placed in a suitable transport medium in ice or liquid nitrogen, transport media include: Hanks balanced salt solution, cell culture media, Phosphate buffered and tryptose-phosphate broth, veal infusion broth and sucrose-phosphate buffered, they should be supplemented with protein, such as bovine serum albumin (BSA) or gelatin, to a concentration of 0.5- 1% to stabilize the viruses (Webster *et al.,* 2002). Medium should be containing antibiotics, which varied according to local condition e.g.: Penicillin-2,000units/ml; Streptomycin- 2mg/ml; Gentamycin-50µg/ml and Mycostatin-1,000units/ml for tissue and tracheal swabs, but five-fold higher concentration for faeces and cloacal swabs, pH of the media should be adjusted to 7.0-7.4 following the addition of the antibiotics (Alexander, 2000).

1.11.3.1.2 Isolation of influenza viruses

Isolation of IAVs in embryonated eggs or cell cultures (Madin-Darby Canine Kidney Cells – MDCK-) along with subsequent identification by immunologic (HA; HI; AGID and immunofluorescence) or genetics techniques -PCR- or by electron microscopy are standard methods for virus diagnosis. Most avian viruses grow readily in embryonated eggs, it is recommended for AI virus isolation (Alexander, 2000). For other animals a combination of embryonated egg and cell culture is desirable (Webster *et al.,* 2002).

1.11.3.1.3 Serum collection

3-5 ml. of whole blood should be taken after onset of clinical symptoms or during convalescent phase or for serological surveillance; allowed to clot and then centrifuged to separate the RBCs and serum, serum samples should be stored at -20°C (Webster *et al.,* 2002).

1.11.3.2 Antigen and genome detection tests

1.11.3.2.1 Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR is the molecular techniques to detect viruses in sample even when they are present in a low levels, it facilitate the rapid diagnosis and genetic characterization of IAV (Webster *et al.,* 2002).

1.11.3.2.2 Direct detection of influenza virus by Enzyme Immunoassay

Is rapid detection tests for IAVs in clinical specimens; tracheal, nasal or throat swabs are suitable specimens for these assays, but fecal samples are not suitable. These tests are useful to confirm the presence of IAVs in embryonated egg and cell culture grown material (Webster *et al.,* 2002).

1.11.3.3 Serological tests

A wide variety of techniques may be used for the serodiagnosis of infection due to influenza viruses; also they are useful in epidemiology and immunologic studies i.e. retrospectively, as well as in evaluation of vaccine immunogenicity.

1.11.3.3.1 Agar gel immunodiffusion test (AGID)

An AGID technique is simple and reliable test in chicken and turkey sera, it is completely unreliable for waterfowl as these birds do not produce precipitating Abs, it has fully validated in other avian species. It is very specific but it is of limited sensitivity, so it is used as diagnostic tool on a flock basis to detect acute and convalescent levels of Abs to IAVs group specific antigens, namely the NP and M proteins line (Beard, 1970a). The test can also be used as a group specific test to identify isolates as IAVs (Alexander, 2000). The AGID test is based on the principle of concurrent migration of antigen and antibody toward each other through an agar medium, when the antigen and specific Abs come in contact, they combine to form precipitate that trapped in gel matrix and produces a visible line (Beard, 1970b).

1.11.3.3.2 Enzyme Linked immunosorbent assay (ELISA)

The ELISA technique has been developed in the late 1960s from radioimmunoassay (RIA), it has been used for detection of Abs to a variety of viruses; as it has the advantage of being environmental safe than RIA; since it requires no isotopes, sensitive, rapid, and highly reproducible and provides a quantitative result, it used widely as a technique for serological diagnosis or seroepidemiological survey on various poultry diseases (Masihi and Lange, 1980). The indirect ELISA was used to detect type-specific Abs to IAVs in the sera of bird (Astorga et al., 1994). The blocking ELISA; in which sera were titrated to block the binding of Monoclonal antibodies (MAbs) to distinct epitopes of the virion was successively used for detection of specific Abs to the strain-specific surface antigens (HA) and type-specific internal antigen (NP) produced by chickens (Hinshaw et al., 1990). A double Ab sandwich blocking ELISA, using MAb against influenza A nucleoprotein (NP) was developed to detect Abs against influenza, enables rapid serological diagnosis and is suited for influenza A Ab screening, especially in species which harbor several influenza subtypes (de Boer *et al*., 1990).

Recently Antigen-capture enzyme immunoassay (A double-antibody sandwich ELISA) developed for the detection of IAVs. The relative specificity of the test was 100% and the relative sensitivity was 79% (Davison et al., 1998).

1.11.3.3.3 Hemagglutination (HA) and hemagglutination inhibition (HI) tests

HA and HI test employ inexpensive, readily available reagents and well-established technique have been used for identification and serological diagnosis of IAVs infections since 1941; HA test depending on ability of IAVs to agglutinate RBCs of fowl and some mammalian (Hirst, 1941),

while HI is caused by attachment of antibody to the virus HA antigenic site (Schild and Dowdle, 1975). HI primary measure Ab to HA; It is subtype-specific test and useful in the serodiagnosis of infection of IAVs and as an index of immunity to infection with certain strains of IAVs, the test also have been used for identification of influenza isolate (Hammond *et al.,* 1980).

1.11.3.3.4 Neuraminidase assay and neuraminidase inhibition (NAI) Assay

NAI is a confirmatory test for influenza virus infection in animal and useful for providing full characterization of IAVs (Webster and Campbell, 1972); as the adequate characterization of IAVs requires the identification not only the HA but also of the NA (Aymard-henry *et al.,* 1973).

1.12 Treatment

Treatment for AI has not been effective and prognosis for flocks infected with HPAI is poor. Use of an antiviral drug only approved for use in human, treatment for other species is symptomatic (AVMS, 2006). Among the first used antiviral compounds in treatment of human IAVs infection are the adamantane derivatives, amantadine (1.aminoadamantane hydrochloride), and rimantadine (α-methyl.1-adamantane methylamine hydrochloride), these were recognized as early 1964 (Belshe *et al.,* 1988). Antiviral activity of those compounds by blocking or slowing the virus penetration in to the host cell (M2 ion-channel inhibitors) (Davies *et al.,* 1964), but drug resistant IAVs emerged during treatment with these drugs, as the result of genetic reassortment in the virus HA and M2 (Belshe *et al.,* 1988). Currently available are neuraminidase inhibitors; Oseltamivir (Tami flu) and Zanamivir (Relenza), which they reduce duration of illness in human cases of IAV (AVMS, 2006).

1.13 Control

1.13.1 Biosecurity

All-in, all-out flock management is ideal rule for prevention of all avian diseases, beside thorough cleaning, disinfection of premises, good sanitary measure for personnel and vehicles enter and leave farm. HPAI need rapid depopulation, placement of an area quarantine and intensive surveillance (Davison *et al.,* 1999). In planning biosecurity measures and control procedures, the frequency of Abs and the role of wild birds in the spread of this virus must be taken seriously (Gilchrist, 2005). Establishing a barrier; Physical isolation and bird proofing of poultry house; between wild bird and commercial poultry is a major biosecurity (Bermudez and Stewart-Brown, 2003)

1.13.2 Vaccination

The cornerstone of many avian infectious diseases control program is induction of specific immunity by vaccination with either live or inactivated microorganisms or their products (Stone *et al.,* 1978). Evolution of influenza viruses creates serious problems for vaccine design. The challenge will be to design safe vaccines that can elicit strong immune responses and yet be maximally effective in the wake of antigenic changes during virus evolution (Steinhauer and Holland, 1987).

In some areas of high poultry population densities and/or in situations in which poverty is widespread. It is impossible to impede the spread of AI only with restriction, biosecurity and stamping out. In addition, some areas appear to be particularly at risk for AI due to the presence of migratory flyways - or - some countries are at higher risk of introduction from "reservoir

environments" (e.g. live bird markets). For these reasons, the concepts of emergency vaccination and prophylactic vaccination have made their way in several countries worldwide. The outcome of these campaigns has been different, ranging from eradication to endemicity of the field virus (EFSA, 2005).

Oil emulsion inactivated products are suitable for field use. The vaccination system must allow the DIVA (Differentiating Infected from Vaccinated Animals) concept, either through appropriate serological tests or through unvaccinated sentinels left in the shed. This is primarily to deal appropriately with the flock if it becomes infected. For this reason, vaccination can only be seen as part of a control strategy based on biosecurity, monitoring, controlled marketing and stamping out (SCAHAW, 2003).

Chapter 2

MATERIAL AND METHODS

2.1 Collection of chicken serum samples for detection of antibodies against AI virus

From April to mid-July 2006, 258 serum samples were collected from healthy flocks in Khartoum State. Total number of farms was 12, of which 11 farms were layer and one farm was broiler. Chickens were in different ages, chicken farms distributed as follow: 5 farms in Khartoum North, 5 farms in Khartoum and 1 farm in Omdurman. The birds were raised-up in open houses.

Whole blood (about 3-5 ml) was taken from birds aseptically; allowed to clot and then centrifuged at 1000 rpm for 15 mins to separate the RBCs. Serum samples were labeled and stored at -20°C until used.

2. 2 Virus strains

The A/Mallard/Neth/12100 (H7N3) and Duck/Sing/645.3/97/128 (H5N1) avian IAV (lab. Adapted strains) were kindly provided by WHO reference laboratory for influenza in United Kingdom.

2.3. Preparation of IAV antigen

2.3.1 Inoculation in allantoic cavity

The procedure used was described by Alexander (2000). Both strains of influenza virus were propagated in the allantoic cavity of nine to ten days-old chicken eggs, by inoculating 0.1 ml. of diluted ($^{1}/_{10}$ - $^{1}/_{100}$) infectious allantoic fluid into allantoic cavity. The eggs were incubated at 37°C, after three to four days, eggs were chilled to reduce bleeding and then the allantoic fluid was harvested. Centrifugation was done for the harvested fluids at 1000 rpm for 5 minutes to remove blood and cells. Hemagglutination titers were determined by Hemagglutination test. The antigen was preserved at -20°C in aliquots. Identity of the virus was done by HA and HI tests as described below.

2.3.2 Inoculation in chorioallantoic membranes (CAM)

Ten to eleven days-old embryonated chicken eggs were inoculated with 0.2 ml. of infectious allantoic fluid, with H5N1, onto the (CAM) and incubated at 37°C. The infected membranes were removed after 72 hours, and then ground in 5 ml Phosphate buffer saline (PBS) (Appendix 2) with Pestle and Mortar. The tissue suspension was freezed and thawed three times to disrupt the remaining cells. The debris was packed by centrifugation at 700-1000 g for ten minutes**.** The pellet was discarded and the supernatant was treated with 0.1% formalin at 37°C for 36 hours and centrifuged again before storage at -20°C. Infected chorioallantoic membrane is recognized as being rich in viral NP (Beard, 1970b; and Dowdle *et al.,* 1974).

2.4 Preparation of Hyperimmune Sera

Hyperimmune sera were prepared as described previously by Samadieh and Bankowski (1971). Eight chicks of 6 weeks old white Bovan breed were divided into two group, four chick in each

group, Group I were inoculated intramuscularly (I.M.) with 1 ml. of $^{1}/_{10}$ dilution of A/Mallard/Neth/12100 (H7N3). Group II were inoculated intramuscularly (I.M.) with 1 ml. of $^{1}/_{10}$ dilution of Duck/Sing/645.3/97/128 (H5N1). On the 21st post-inoculation day, each group was given a series of four injections, at five-day intervals between injections, of 0.5 ml. of the same strain of virus preparation. Blood was collected at 14 and 21 days post primary inoculation and two week after last inoculation i.e. at 50 days post primary inoculation, then several collections were made at 65, 69, 74, 78 and 80 days post primary inoculation. The HI titers of the sera were determined by HI test.

2.5. Hemagglutination test

2.5.1 Reagents

Chickens RBCs in Alsever's solution (Appendix 3) or EDTA.

PBS (0.1M) pH 7.2.

Physiological saline, 0.85% NaCl (Appendix 1).

Sterile distilled water.

2.5.2 Preparation of chicken RBCs

The procedure was described by Hierholzer and Suggs (1969) and Webster *et al* (2002).

i. Blood from SAN chickens was collected and stored in an equal volume of the Alsever's solution.

ii. 5 ml of blood was filtered through gauze into conical centrifuge tube.

iii. Centrifuged at 1200 rpm for 10 minute

iv. The buffy layer of white cells and supernatant were aspirated.

v. 50 ml PBS was added and mixed gently.

vi. Centrifuged at 1200 rpm for 10 minute, The buffy layer of white cells and supernatant were aspirated

vii. Repeated PBS wash two times.

viii. RBCs were resuspended to a final volume of 12 ml using a 15-ml graduated conical centrifuge tube.

ix. Centrifuged at 1200 rpm for 10 minute.

x. The volume of packed cells was estimated (each 1 ml of blood gives 0.4 ml) and RBCs were diluted to 1% concentration.

2.5.3 Hemagglutination procedure

This was applied as described by Alexander (2000).

i. 25 µl of PBS was dispensed into each well of a plastic U shaped-bottomed micro titer plate. 25 µl of PBS was dispensed into row H, which was RBCs control row.

ii. 25 µl of infected allantoic fluid was dispensed into first well (A1-F1), series of two-fold dilution were made.

iii. 25 µl of PBS was dispensed into each well, followed by 25 µl of chicken RBCs.

iv. Plate was mixed by tapping and RBCs allowed for settling for 40 minutes at room temperature. Control RBCs in last row should be settled to distinct button.

v. Hemagglutination was determined by observing the presence or absence of tear-shaped streaming of RBCs, and 1 Hemagglutination unit (HAU) was determined.

2.6 Hemagglutination inhibition test

2.6.1 Reagent

Influenza Type A antigens (H7N3), and (H5N1), and prepared antisera for both virus strains.

Chickens RBCs in Alsever's solution or EDTA.

PBS (0.1M) pH 7.2.

Physiological saline, 0.85% NaCl.

Sterile distilled water.

2.6.2 Hemagglutination inhibition test procedure

Test was performed according to Alexander, (2000).

i. 4 HAU of virus was determined by Hemagglutination test.

ii. 25 µl of PBS was dispensed into each well of a plastic U-bottomed micro titer plate.

iii. 25 µl of tested sera and controls sera was placed in appropriate wells. Series of tow-fold dilutions were made across the plate.

vi. 25 µl of 4 HAU of virus was dispensed into each well of the plate. Leaved for 30-40 minutes at room temperature.

iv. 25 µl of chicken RBCs was dispensed into each well, and plate was mixed by tapping and RBCs allowed for settling for 40 minutes at room temperature. Control Sera checked, negative control serum should not give a titer more than 2^2 and positive control serum for which the titer should be within one dilution of known titer.

v. The HI titer is the highest dilution of serum causing complete inhibition of 4 HAU of antigen. HI titers regarded positive if there is inhibition at initial serum dilution of 2^4 or more against 4 HAU of antigen.

2.7 Agar gel immunodiffusion test-AGID

2.7.1 Reagents

Formalin inactivated antigen from infected CAM.

Hyperimmune serum as positive control.

Negative antiserum.

1% Purified agar.

Sodium chloride (NaCl).

Phosphate buffer PB.

Distilled water.

Phenol.

1% Sodium dodecyl sulfate (SDS).

2.7.2 Agar gel medium

Two Formulas for Agar were made:

First formula: The agar gel was prepared as described by Samadieh and Bankowski (1971); and Webster *et al* (2002) as follows: NaCl 8%, Agar 1%, and Phenol 0.5%., in PB.

Second formula: The agar gel was prepared as follows: NaCl .85%, Agar 1%, and Phenol 0.5%., in distilled water as described previously by Ferreira *et al.,* (2002).

The agar mixture was dissolved by boiling on microwave oven, and then mixed by swirling after removing from the microwave. Then agar was allowed to cool at room temperature for 10 to 15 minutes after dispensing into Petri dishes.

2.7.3 AGID test procedure

The test was performed as it was described by Webster *et al* (2002).

i. 15-17 ml. of melted agar were dispensed into a 100 × 15 mm Petri dishes, with agar thickness of approximately 2.8 mm.

ii. Dishes were allowed to cool in a relatively dust-free environment with lids off for 15-30 minutes. Agar was cut, after it had hardened, using a template with wells of 5 mm diameter, in a hexagonal pattern of six peripheral wells for serum samples and a central well for antigen.

iii. IAV antigen was placed in central well after it was mixed with equal volume of SDS. Positive and negative controls and tested sera were placed in peripheral wells.

iv. Petri dishes were covered after filling and incubated at room temperature in humid chamber. Petri dishes were examined after 24 and 48 hours of incubation. The appearance of one or more clearly definable precipitation lines before or at 48 hours constituted a positive test result. Absence of any precipitation lines was recorded as a negative test result.

2.8 ELISA test

The ELISA kit was obtained from IDEXX laboratories, United Kingdom. This kit was used for detection of Ab to IAV in chicken serum samples.

2.8.1 Reagents

IAV coated plates.

IAV positive - diluted chicken anti-IAV, preserved with sodium azide.

Negative control - diluted chicken serum non-reactive for anti-IAV Preserved with sodium azide.

Goat anti-chicken/Goat anti-turkey Horseradish peroxidase conjugate, preserved with gentamycin.

Sample Diluent buffer preserved with sodium azide.

TMB Substrate.

Stop solution.

2.8.2 Preparation of serum samples

Each tested sample was diluted five hundred fold ($^{1}/_{500}$) with sample diluent prior to being assayed.

2.8.3 ELISA test procedure

i. 100 µl of undiluted negative control were dispensed into two adjacent wells (A1 and A2).

ii. 100 µl of undiluted positive control were dispensed into two adjacent wells (A3 and A4).

iii. 100 µl of diluted sample were dispensed in appropriate wells. All samples were run in duplicate.

iv. Plate was incubated for 30 minutes at room temperature.

v. Each well was washed with approximately 350 µl of distilled water for 3-5 times.

vi. 100 µl of Goat anti-chicken/ Goat anti-turkey Horseradish peroxidase conjugate were dispensed in all wells.

vii. Plate was incubated for 30 minutes at room temperature.

viii. Each well was washed with approximately 350 µl of distilled water 3-5 times.

ix. 100 µl of TMB Substrate were dispensed in all wells.

x. Plate was incubated for 15 minutes at room temperature.

xi. 100 µl of Stop solution were dispensed in each well.

xii. Optical density was measured at 650nm.

2.8.4 Calculations

$$\text{Negative Controls mean (NCx)} = \frac{\text{OD of first well + OD of second well}}{2}$$

$$\text{Positive controls mean (PCx)} = \frac{\text{OD of first well + OD of second well}}{2}$$

$$\text{Sample/positive (S/P Ratio)} = \frac{\text{Sample Mean - NCx}}{\text{PCx-NCx}}$$

2.8.5 Interpretation of results

The assay be valid when, negative control mean (NCx) wells had an optical density at 650nm (OD_{650}) of < 0.150, and a differences between the positive control mean and negative control (PCx-NCx) is greater than 0.075. The relative level of Ab in the unknown is determined by calculating the sample to positive ratio (S/P). Serum samples had S/P ratios of less than or equal to 0.5 was considered negative and S/P ratios greater than 0.5 was considered positive and indicate exposure to IAV.

Chapter 3

RESULTS

3.1 Growing of AIV in embryonated eggs

3.1.1 Inoculation in allantoic cavity

Infected allontoic fluid, with H5N1 and H7N3, agglutinated chicken RBCs. Hemagglutination titers were determined by Hemagglutination test gave titer range between 2^6-2^{11} to H7N3 and 2^6-2^{10} to H5N1.

3.1.2 Inoculation in chorioallantoic membranes (CAM)

Infected chorioallantoic membrane revealed hemorrhage and thickening. Clear precipitin line was developed between IAV known antisera and prepared antigen

3.2 Quantitation of AIV antibodies in hyper-immune sera

ELISA, AGID and HI tests were conducted for the prepared hyper-immune serum. Results are shown in Table 1 and Table 2. The relationship between HI and ELISA was determined by measuring regression (R^2), which was found 0.44 and 0.389, for group (1) and (2) respectively (figure 1, 2).

3.3 Sero epidemiology of AI virus antibodies

3.3.1 Agar gel immunodiffusion test

3.3.1.1 Validation and performance of the test

No precipitin line was developed between different IAV prepared antisera and prepared antigen when the concentration of sodium chloride (NaCl) in the agar medium was 8%. But when the concentration of NaCl was decreased to 0.85% well-defined precipitin lines were developed between the prepared antigen and hyper-immune Sera, while no precipitin line was developed between antigen and normal serum (figures 3, 4). Accordingly the second formula was used throughout the research work to test all chicken sera

3.3.1.2 Results of tested sera

258 sera were tested using AGID test, 167 sera were positive (64.73%), 81 sera were negative and 10 sera were suspicious in their results. Sera collected from chickens from Omdruman gave 86% seropositivity and thus come first then Khartoum North with 64.29% seropositivity followed by Khartoum region with 54.17% seropositivity (Table 3). Relationship between age and number of positives was shown in Table 4 and figure 3 (R^2 equal 0.8). Relationship between the date and place of sampling on one hand, and AGID results are shown in Table 5, and indicate that the high percentage of positives was in June (80.33%).

Table 1: Group -I- ELISA, AGID and HI tests for detection AI antibodies in chicken hyperimmune sera inoculated by H7N3

Days after first inoculation	ELISA (OD)	AGID results (ppt lines)	HI titers
0	0.05	-	0
14	2.353	+	32
→ 21	0.664	+	64
↔ 50	3.229	+	1024
65	3.130	+	1024
69	3.150	+	512
74	2.979	+	512
78	2.181	+	512
80	2.074	+	512

→ Starting booster doses, 4 doses with interval 5 days between them.

↔ Two weeks after last booster dose.

OD: Optical density.

ppt: precipitation.

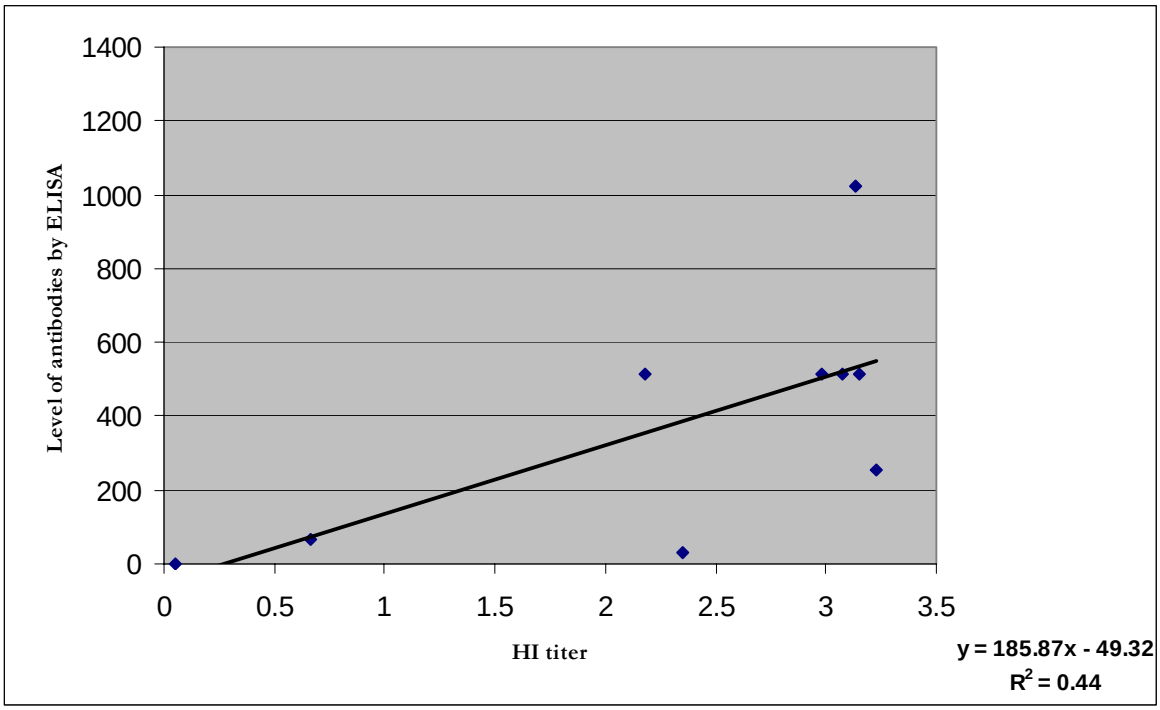

Figure 1: Correlation between HI and ELISA tests for detection AI antibodies in chicken hyperimmune sera inoculated by H7N3.

Table 2: Group -2- ELISA, AGID and HI tests for detection AI antibodies in chicken hyperimmune sera inoculated by H5N1

Days after first Inoculation	ELISA (OD)	AGID results (ppt lines)	HI titers
0	0.05	-	0
14	1.079	+	64
→ 21	0.894	+	128
↔ 50	2.333	+	1280
65	1.979	+	768
69	2.457	+	512
74	2.914	+	416
78	2.653	+	512
80	2.679	+	512

→ Starting booster doses, 4 doses with interval 5 days between them.

↔ Two weeks after last booster dose.

OD: Optical density.

ppt: precipitation.

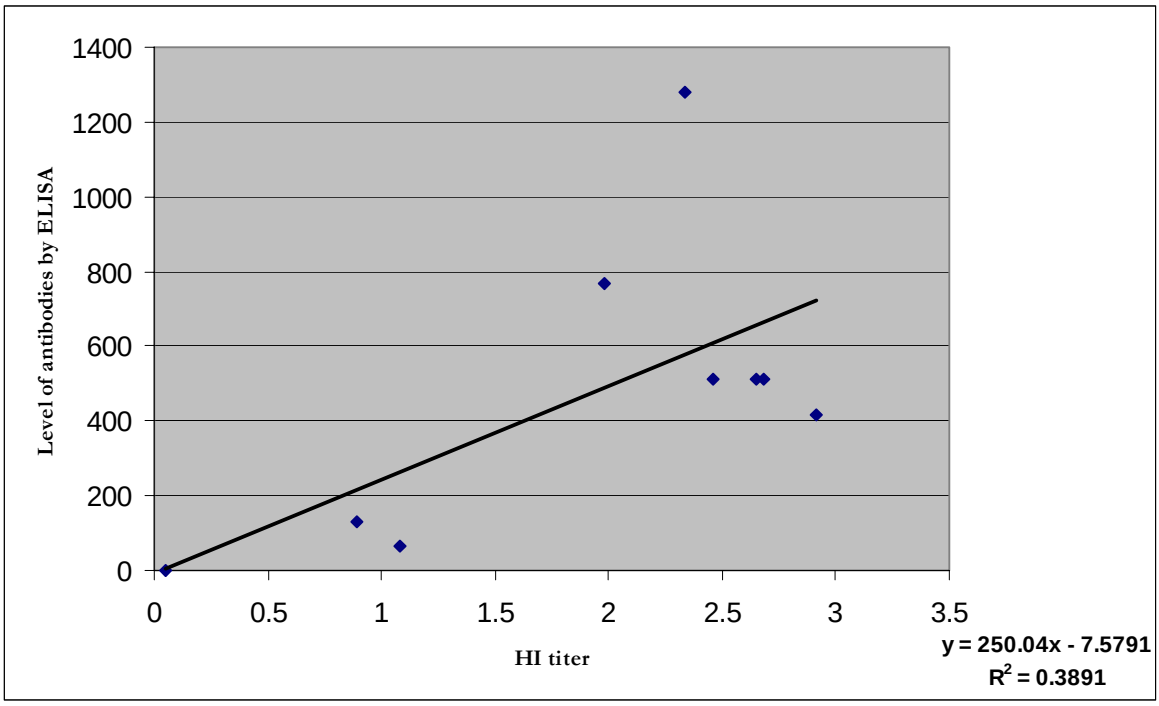

Figure 2: Correlation between HI and ELISA tests for detection AI antibodies in chicken hyperimmune sera inoculated by H5N1.

Table 3: AGID test results for detection AI antibodies in chicken sera collected from 3 areas at Khartoum State by

Region	Result of AGID test			
	Positive (%)	**Negative**	**Suspicious**	**Total tested**
Khartoum North	72 (64.29)	35	5	112
Khartoum	52 (54.17)	40	4	96
Omdruman	43 (86)	6	1	50
Total	**167 (64.73)**	**81**	**10**	**258**

The numbers between brackets are percentage of positive.

Table 4: Effect of age on the prevalence of AIV antibodies in various poultry farms detected by AGID test

	Result of AGID Test			
Age of Flock	**Positive (%)**	**Suspicious**	**Negative**	**Total tested**
3 months	13 (61.9)	-	8	**21**
4 months	6 (54.5)	-	5	**11**
6 months	15 (71.4)	-	6	**21**
7 months	38 (50.7)	6	31	**75**
9 months	7 (58.3)	2	3	**12**
11 months	15 (75)	1	4	**20**
14 months	30 (62)	-	18	**48**
17 months	43 (86)	1	6	**50**
Grand Total	**167**	**10**	**81**	**258**

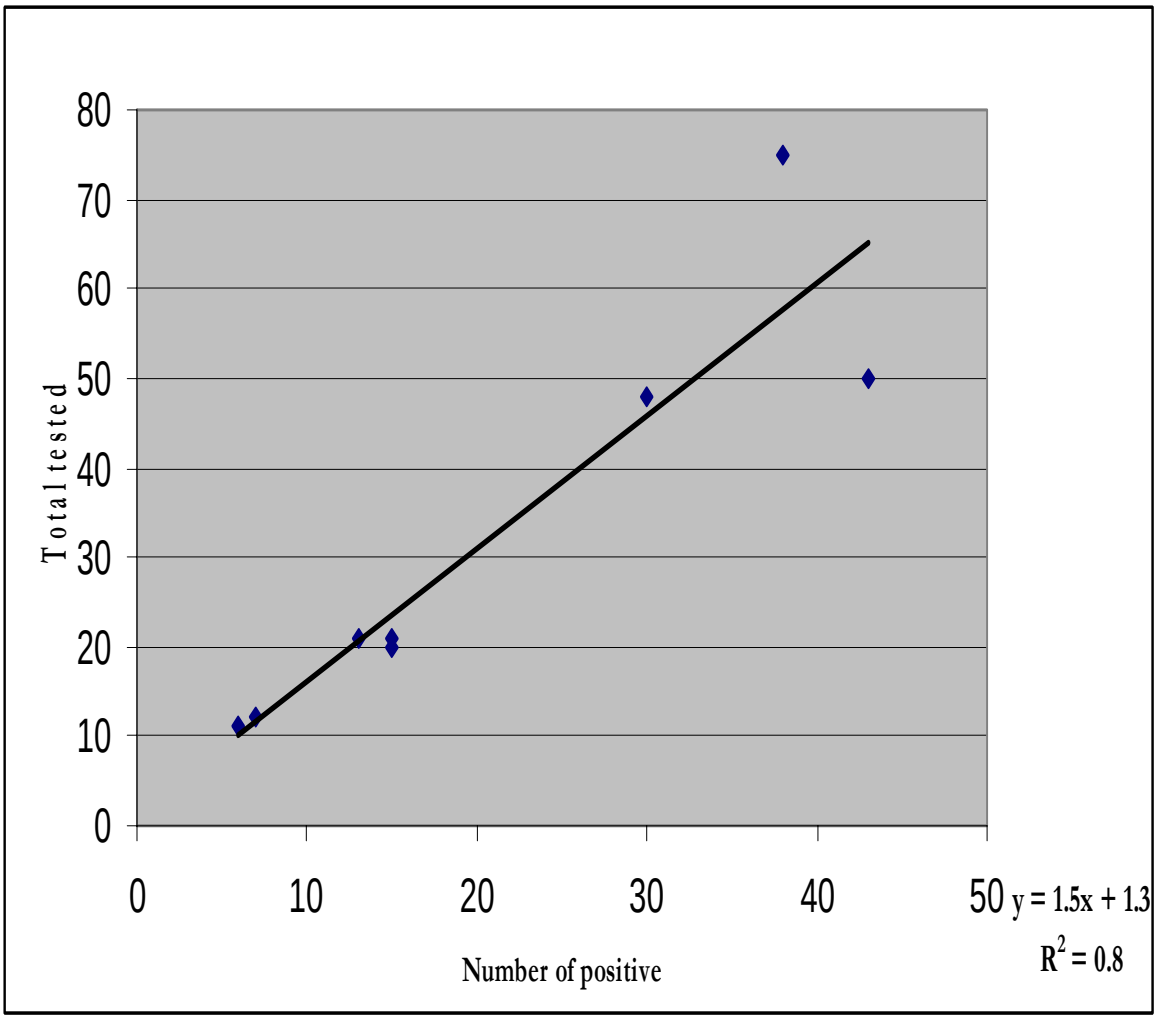

Figure 3: Relationship between age and number of positives on using AGID test.

Table 5: Relationship between place, collection date and number of positives on using AGID test

2006									
Month	**April**		**May**			**June**		**July**	
Date	22	30	2	21	22	4	29	16	
Place*	KN	K	K	K	K	K	O	KN	
Total Number of Samples	12	22	30	12	21	11	50	100	
Number of Positive	7	8	17	8	13	6	43	65	
Percentage of Positive	44.12%		60.32%			80.33%		65%	

* KN, Khartoum North. K, Khartoum. O, Omdurman

Figure 4: Two precipitation lines produced by hyperimmune sera to AIV H5N1 on AGID test.

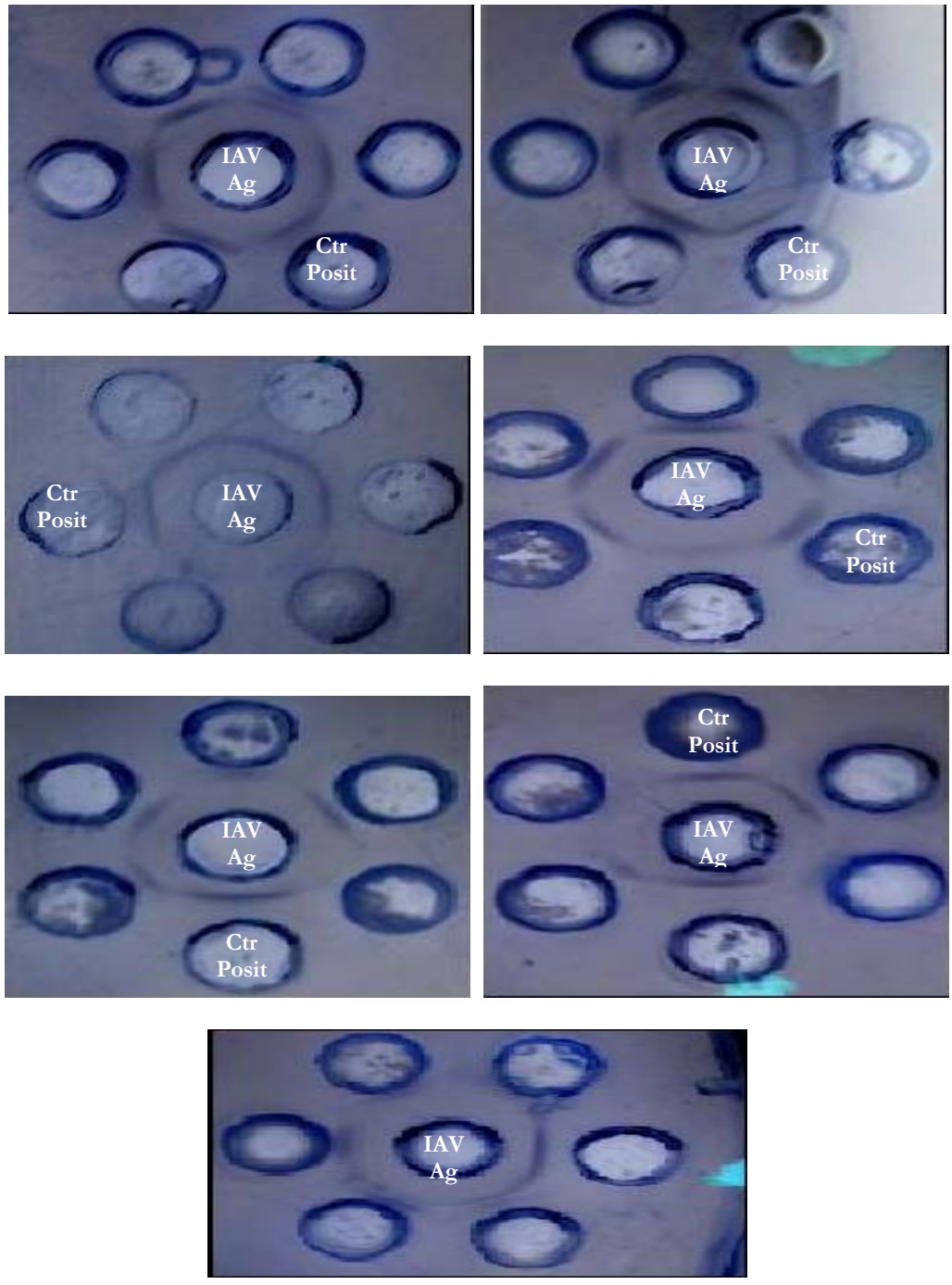

Figure 5: Pattern of AGID test results of testing serum samples for antibodies against IAV.

3.3.2 HI test

The Ab titers were expressed as the reciprocal of the highest dilution of the serum at which complete inhibition of HA was seen, titer 2^4 or more was considered positive. The increase of Ab titers of hyperimmune sera was determined (Table 1 and Table 2). Field sera revealed no positive results except 3 sera which gave titers of 16-32 with AIV H7N3.

3.3.3 ELISA test

3.3.3.1 Validation and performance of the ELISA test

AI virus Ab test kit was obtained from IDEXX laboratories. The test was performed according to manufacturer's instructions. The ELISA reactions were read with spectrophotometer at the suggested wavelengths (650nm). Each test result was included in this study only if the assay was declared valid according to the specifications of the manufacturer. For the assay to be valid, negative control mean (NCx) wells had to have an optical density at 650mn (OD_{650}) of < 0.150 (Table 6 and figure 6), the differences in OD between the positive control mean (Table 7 and figure 7) and negative control (PCx-NCx) should be greater than 0.075 (Table 8 figure 8).

3.3.3.2 Result of tested sera

192 Sera were tested by ELISA, 60 (31.23%) of which were positive, distributed as Omdruman 38.89%, Khartoum North 33.33% and Khartoum region 25.33% (Table 9 and figure 9). Table 10 and figure 10 indicate relationship between region and level of Abs, Sera collected from

Omdruman had high level of Abs which may be due age of flock (17 Months), the relationship between age of flock and titer of Abs was shown in Table 11 and figure 11. Correlation between mean of antibodies level and flock age was illustrated in figure 12 where regression (R^2) was calculated 0.7. Relationship between the place of sampling, collection date, and number of positive sera shown in Table 12 and figure 13, indicate that the high percentage of positive in April (36.67%). Positive flocks had 4-14 sero-positive chickens with percent of positive range from 23.7 - 50%; two flocks had no positives.

3.4 Comparison between ELISA and AGID

Based on the 192 randomly selected sera, comparison between ELISA and AGID was done (Table 13). The percentage of total Positive agreement was too low 42.15%, while the percentage of total negative agreement was relatively high 87.30% (Table 14).

Table 6: Absorbance values for standard negative controls sera for ELISA test

Plate number	First well absorbance value	Second well absorbance value	Mean of absorbance value	Standard deviation (SD)
1	0.070	0.062	0.066	0.006
2	0.065	0.068	0.067	0.002
3	0.086	0.072	0.079	0.010
4	0.059	0.064	0.062	0.004
5	0.091	0.067	0.079	0.017

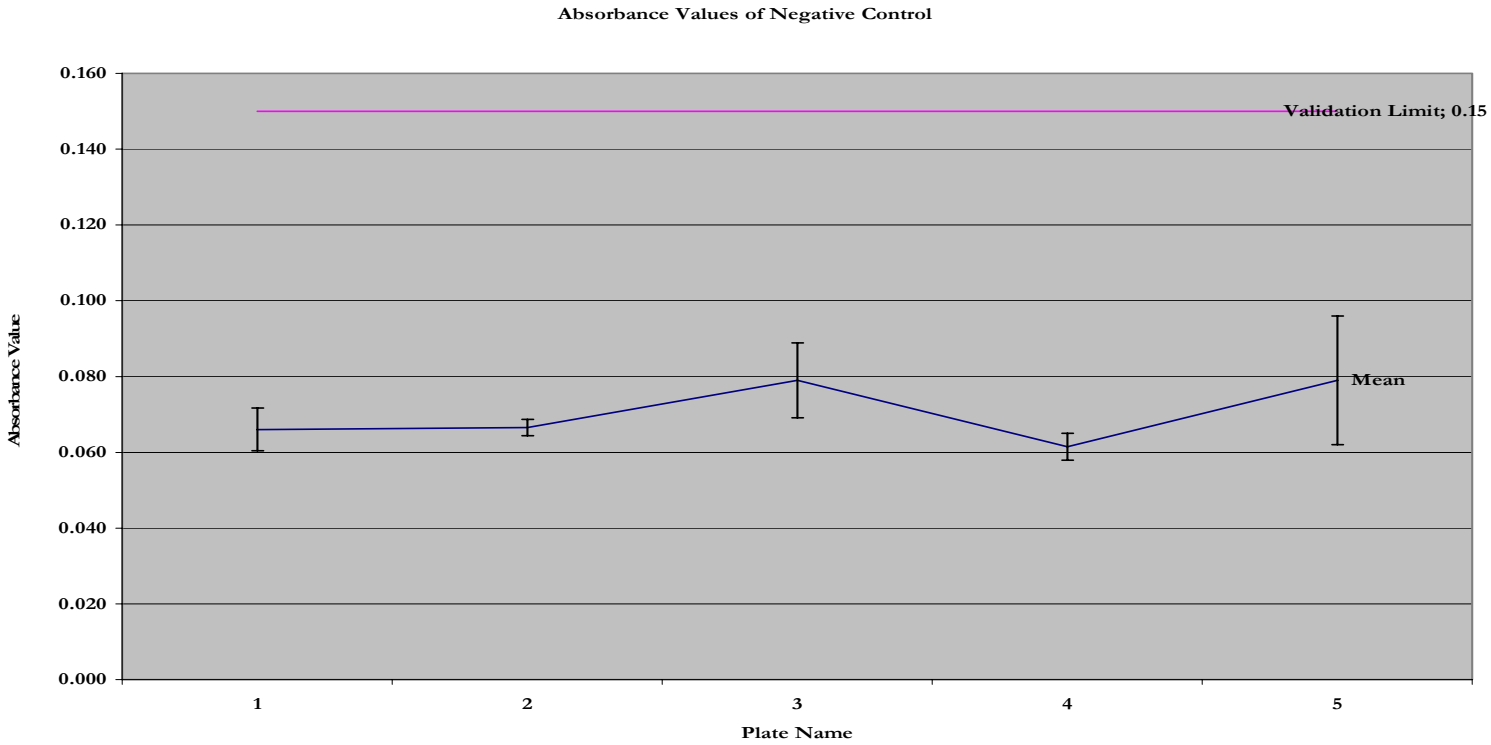

Figure 6: Absorbance values of negative control sera
Line at the bottom indicates means spectrophotometer reading for standard negative controls, while upper line indicates the validation limit.

Table 7: Absorbance values for standard positive controls sera for ELISA test

Plate number	First well absorbance value	Second well absorbance value	Mean of absorbance value	Standard deviation (SD)
1	0.636	0.604	0.620	0.02
2	0.723	0.664	0.694	0.04
3	0.717	0.664	0.691	0.04
4	0.612	0.552	0.582	0.04
5	0.642	0.645	0.644	0.00

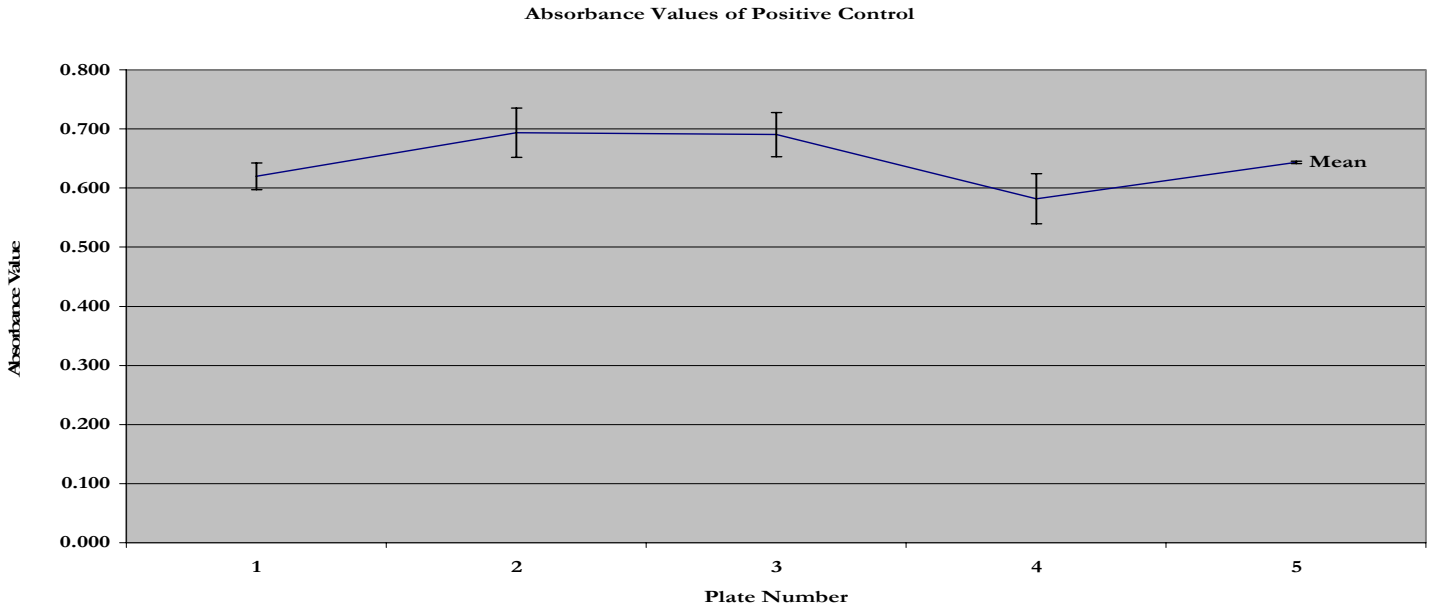

Figure 7: Absorbance values of positive control.
Line indicates means spectrophotometer reading for standard positive controls.

Table 8: Means absorbance value for standard positive and negative controls sera and their difference for ELISA test

Plate number	Mean of absorbance for Positive control	Mean of absorbance for negative control	Difference (PCx-NCx)
1	0.620	0.066	0.554
2	0.694	0.067	0.627
3	0.691	0.079	0.612
4	0.582	0.062	0.520
5	0.644	0.079	0.565

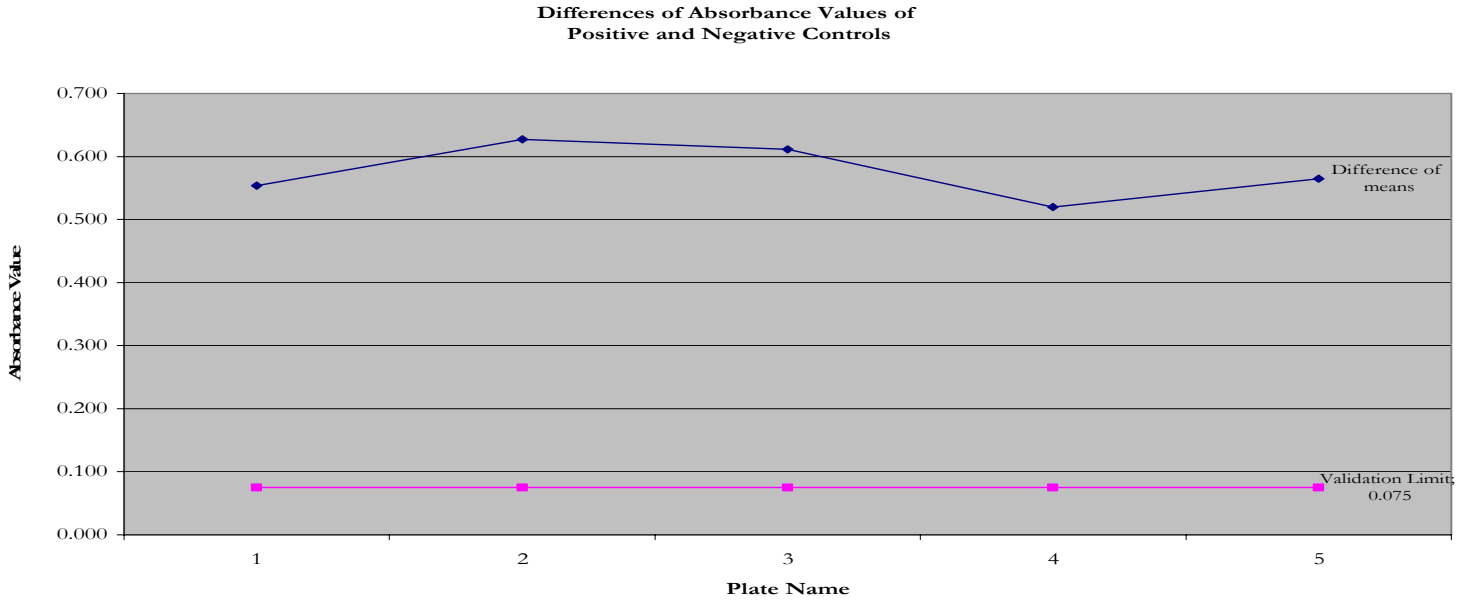

Figure 8: Differences of absorbance value of positive and negative controls sera.
Upper line indicates the differences between optical density Mean of positive and negative standard controls. Bottom line indicate validation limit.

Table 9: Prevalence of AIV antibodies in various Poultry farm at Khartoum state tested by ELISA test

Region	Total Number of Sample	Number of farm	Number of positive	Percentage of Positive
Khartoum North	81	5	27	33.33 %
Khartoum	75	5	19	25.33 %
Omdruman	36	1	14	38.89 %

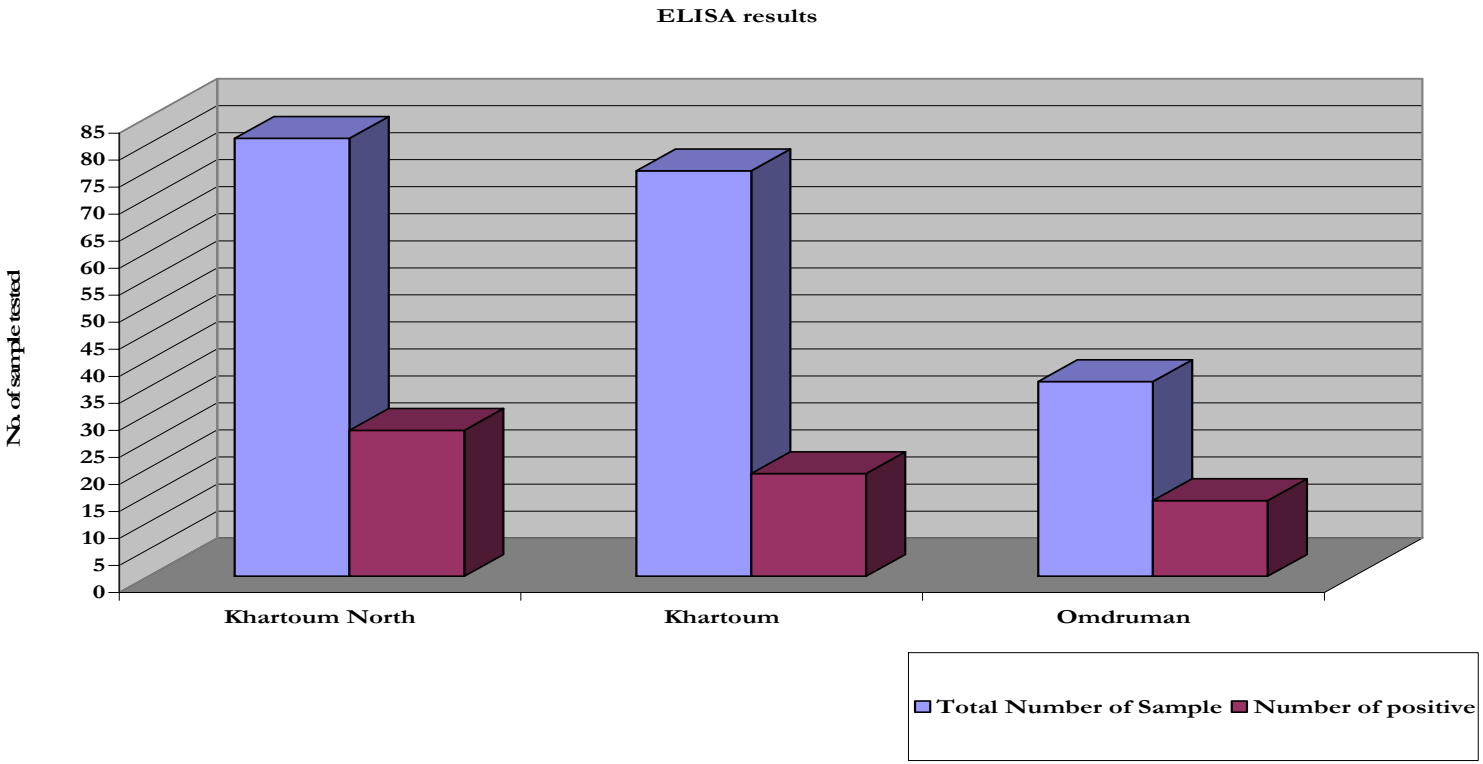

Figure 9: Prevalence of AIV antibodies in Khartoum State.
Area with their number of samples and positives.

Table 10: Relationship between region and level of antibodies for AI by ELISA test

Region	Mean of antibodies (S/P) ±SD
Khartoum North	0.41 ± 0.3
Khartoum	0.36 ± 0.27
Omdruman	0.53* ± 0.9

* Age of flock 17 months.

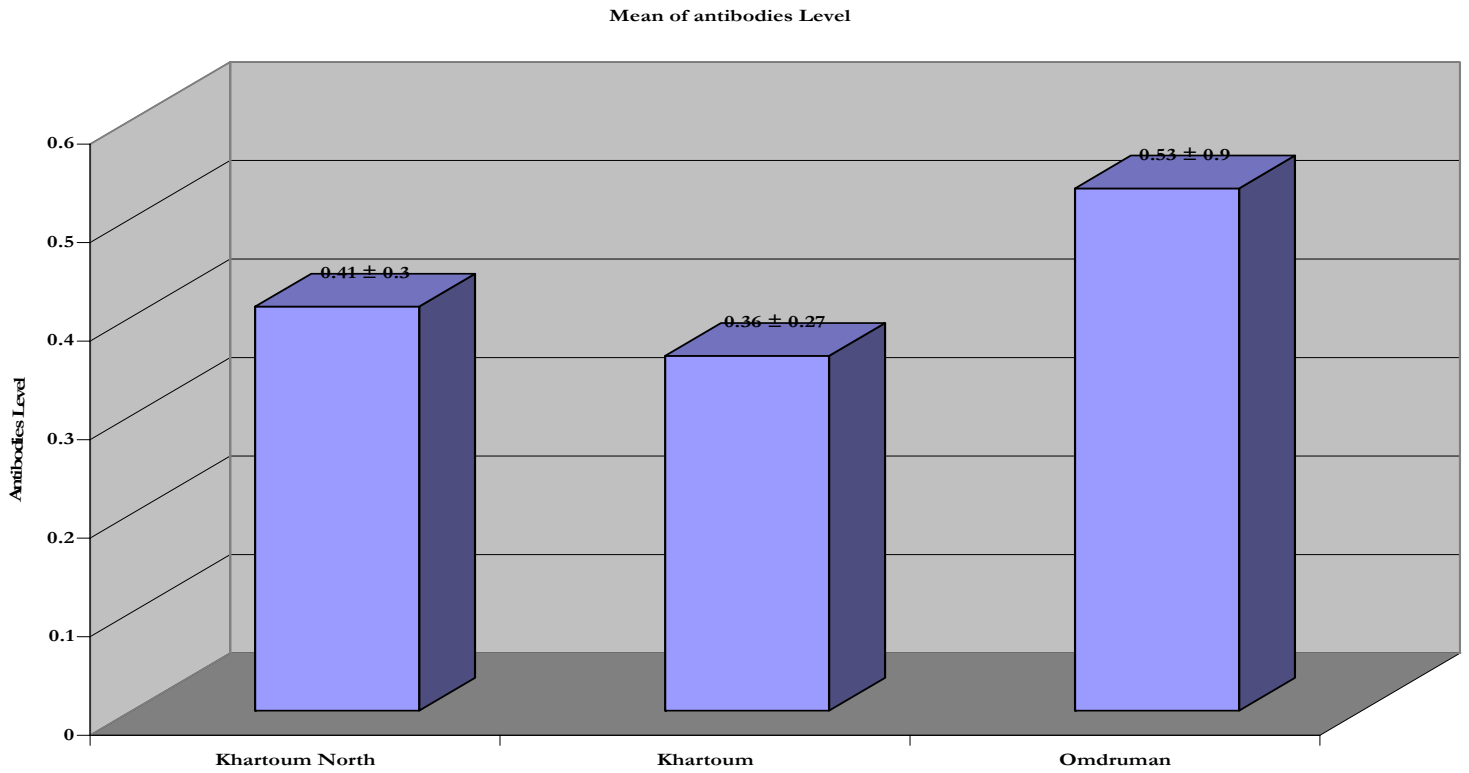

Figure 10: Mean of Antibody level for AI in sera collected from 3 areas in Khartoum State.

Table 11: Effect of age on the prevalence of antibodies level as detected by ELISA test

Age of Flock	Mean of Antibody Level ±SD
17 Months	0.53 ± 0.9
14 Months	0.49 ± 0.31
9 Months	0.49 ± 0.25
7 Months	0.44 ± 0.29
6 Months	0.36 ± 0.19
4 Months	0.16 ± 0.09
3 Months	0.16 ± 0.1

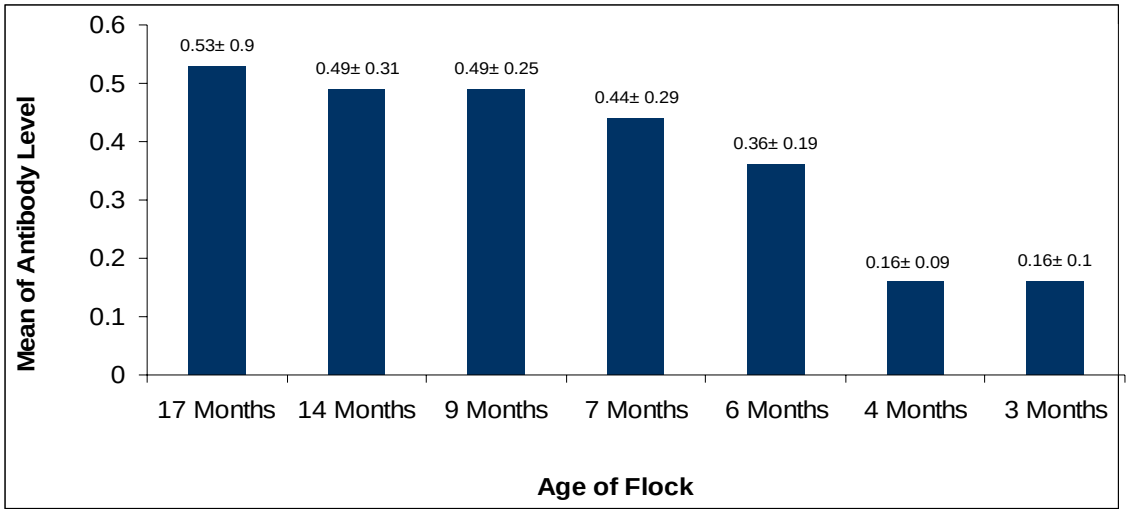

Figure 11 Effect of age on the prevalence of antibodies level as detected by ELISA test.

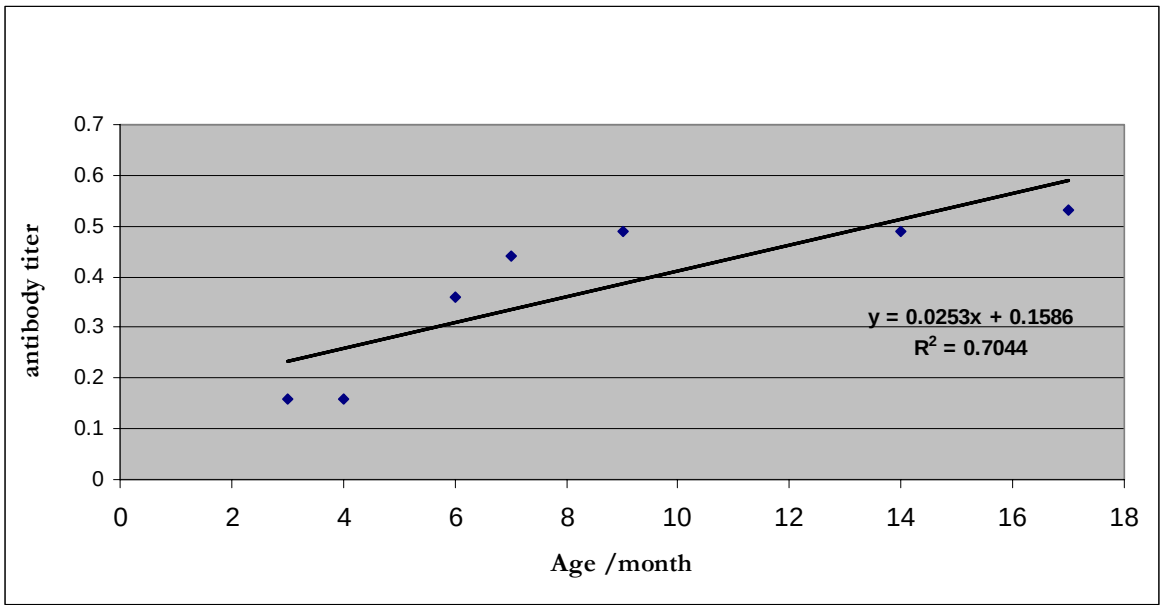

Figure 12: Correlation between mean of antibodies level and flock age.

Table 12: Relationship between place and collection date and number of positives using ELISA test for detection of AIV antibodies

2006									
Month	**April**		**May**			**June**		**July**	
Date	22	30	2	21	22	4	29	16	
Place*	KN	K	K	K	K	K	O	KN	
Total Number of Samples	10	20	20	10	15	10	36	71	
Number of positive	5	6	8	5	0	0	14	22	
Percentage of positive	36.67%		28.89%			30.43%		30.99%	

* KN, Khartoum North. K, Khartoum. O, Omdruman.

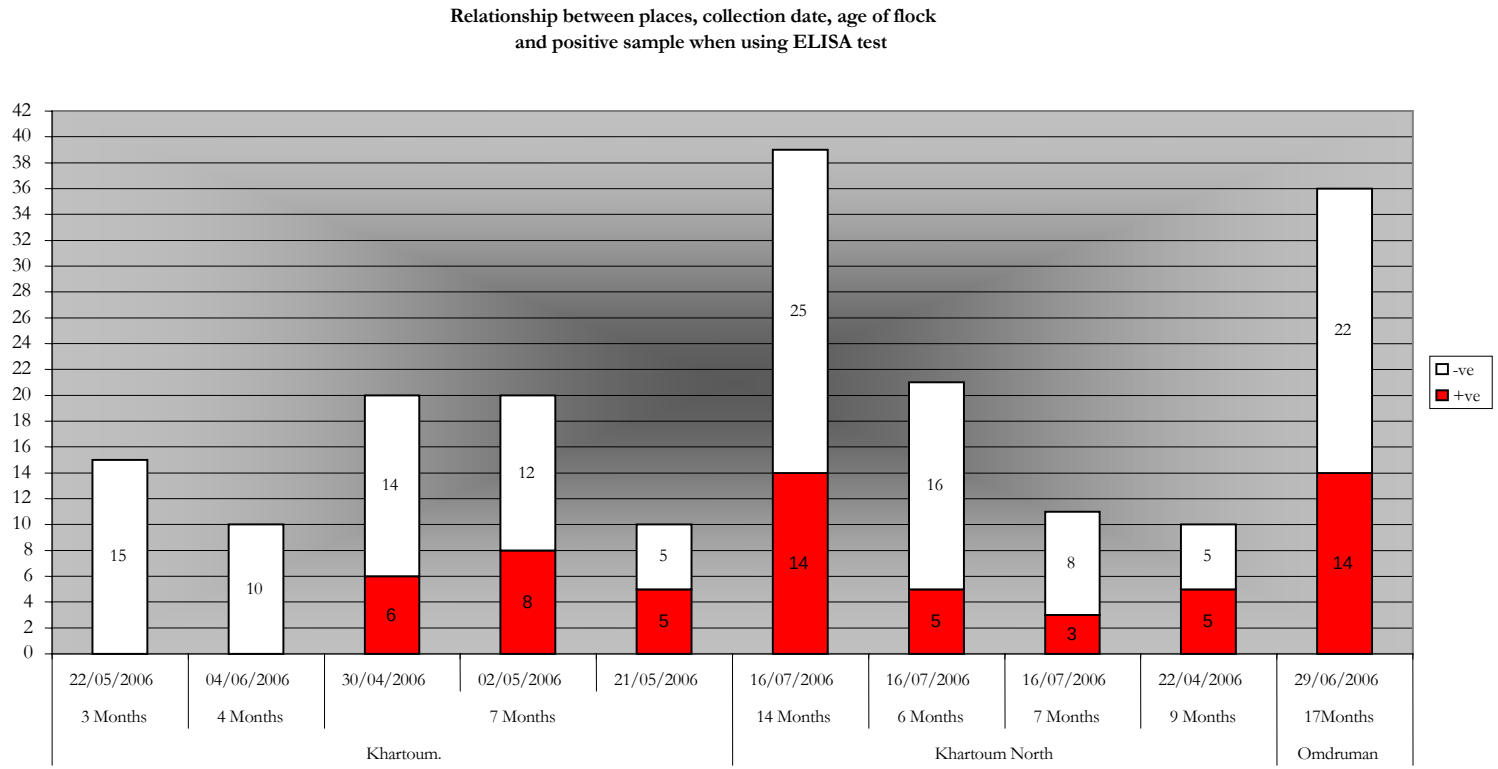

Figure 13: Relationship between Place, collection date, age of flock and positive samples when Using ELISA test for detection of AIV antibodies.

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Table 13: Comparison between the ELISA and AGID tests for detection of AIV antibodies

AGID	ELISA		
	Positive	**Negative**	**Total**
Positive	51	70	**121**
Negative	8	55	**63**
Suspicious	1	7	**8**
Total	**60**	**132**	**192**

Table 14: Percentage of total negative and positive agreement for field sample between ELISA and AGID tests

Percentage of total Negative agreement (%)	Percentage of total Positive agreement (%)
87.30	42.15

Chapter 4

DISCUSSION

Global forces aimed to eradicate the currently circulating strains of HPAI. Since a portion of the current surveillance and eradication efforts rely on serologic surveys, as IAVs share a common internal antigenic component (Schild and Pereira, 1969); and isolation of the causative agent from birds having mild respiratory signs is not always possible, and the procedures are troublesome and time consuming to do. Therefore, a rapid technique to identify the presence of any of the strains of IAV in the flock is of importance in diagnosis. This study compared serological tests for the detection IAVs Abs in chicken sera.

The prevalence of AI in Khartoum state was studied among some farms. This survey confirmed the presence of AI in Khartoum state; that had been shown by positive results when AGID and ELISA tests were conducted to verify the presence of Abs against AI, Similar results was obtained by Elamin (2000) who reported detection of Abs against AI in chicken sera obtained from Khartoum state by AGID and ELISA tests. This is also in agreement with Kamaleldein (2007) who demonstrated the presence of Abs against AI in the same area when used AGID test. Omdruman was found to have high percent of positive sera when AGID and ELISA tests were conducted, which could be due to age of the flock; since all sera were collected from hens aged 17 months. AGID results illustrated that, the number of positive sera/flock strongly correlated with flock age, see figure (3) (R^2=0.8). Also the Ab level when detected by ELISA is correlated with the flock age (R^2=0.7), see figure (11 and 12). The percentage of total positive agreement between AGID and

ELISA tests was found too low when these tests were conducted in parallel, while the percentage of total negative agreement was found relatively high.

Negative results in HI test may mean either the absence of influenza antibodies or the presence of antibodies against a different strain, antigenically distinct from those viruses used in HI test (Beard, 1969). During the course of this study, positive sera found by AGID and ELISA was tested by HI test to identify subtype; firstly was tested against Duck/Sing/645.3/97/128 (H5N1) strain. No inhibition was occurred; in spite of large number of positive sera for type specific antibodies against IAV were detected by AGID and ELISA. Failure of HI test to detect antibodies could be due to the presence of antibodies against a different strain, antigenically distinct from the strain used in HI test. Secondly a few sera (3 sera) were inhibit agglutination of the A/Mallard/Neth/12100 (H7N3) strain with very low titer (2^4 and 2^5) when they were tested by HI test, this is in consistence with Kamaleldein (2007), who demonstrated the presence of Abs against subtype H7 in chicken sera collected from Khartoum state, or it may be explained as cross-reaction due to homologous NA antigen which could give low degree of cross-reactivity with other HA subtypes due to homology with the NA antigen (Schild and Dowdle, 1975). This cross-reactivity is generally not higher than 1:16 (2^4) and not detected with another IAV strain with different NA. Elamin (2000) was able to identify the subtype (H3) for 6.5% of the isolates of the influenza viruses that circulate during that period and caused outbreak in a farm in Khartoum North area during the winter season of 1998, where as Ali and Kheir (2007) succeed to isolate H5N1 during the outbreak occurred in 2006. In contrast Kamaleldein (2007) detected the presence of Abs against subtype H7 in chicken sera collected in Khartoum state. This illustrated that some strains of influenza type A may be circulated in poultry population in Khartoum state.

In the present study the immunodiffusion test revealed well-defined precipitation line between antiserum of IAV and the prepared corresponding IAV antigen. The precipitation line was identical for both antigenic strains of IAV antiserum, due to presences of a common NP antigen in all IAV strains, which was confirmed previously by Hana and Hoyle (1966). Two precipitation lines were observed between influenza antigen and some of hyperimmune sera, see figure (4). This observation was previously reported by group of investigator when using sodium dodecyl sulfate (SDS) (Schild and Pereira, 1969), owing these results to existence of NA molecule in the virus particle which was broken down by SDS into two separate components each capable of reacting with antiserum, while HA was broken down in the presence of SDS into structures incapable of reacting with antiserum. Other investigators used Sodium deoxycholate (Hana and Hoyle, 1966) and Ether (Samadieh and Bankowski, 1971) to disrupt IAV; they obtained four lines and a single line respectively. These results indicate that the choosing of appropriate disrupting agent should be carefully considered to minimize disintegration of NP and, in effect, increasing the sensitivity of the test (Samadieh and Bankowski, 1971). Also Schild, (1972) demonstrated that, the two distinct precipitation lines could be formed when a mixture to anti-M and anti-NP were used to react with IAV antigen disrupted by SDS.

Anti-influenza Abs was detected in sera of all AI virus-inoculated chickens by day 14 postinoculation (PI), when AGID test was conducted. This result is in agreement with results obtained by Beck *et al* (2003) who found that sera from all vaccinated hens were positive by day 14 PI when AGID and ELISA tests were used and differed from Beard (1969) and Snyder *et al* (1985) findings, who showed that AGID test was positive with chicken sera for at least 8 weeks postinoculation, the later suggested that infecting dose may be too low (0.1 ml intratracheal) to

induce precipitating antibody, or the low pathogenic subtype (H9N2) employed did not replicate in vivo to the extent of providing the necessary antigenic stimulation for the elicitation of precipitation antibody. Samadieh and Bankowski (1971) attributed the differences in times of appearance of precipitating antibodies to the defense mechanism. Since one day chicks are immunologically incompetent, active production of Abs was not detected until 3 weeks after inoculation.

The results demonstrated a high degree of correlation between the AGID and ELISA test results when prepared hyperimmune sera were tested by both tests. Our results are similar to those obtained by Shafer et al. (1998) who demonstrated a high degree of correlation between the AGID and ELISA test results. No correlation between HI and ELISA tests was found, R^2 equal 0.44 and 0.389 for group (1) and (2) respectively this is in parallel to Hinshaw, *et al.* (1990) study, in which the authors indicated that the high levels of antibodies to the NP differed in the level and specificity of antibodies to the epitopes on the HA produced by chickens immunized against certain strains of IAV. Conversely, close correlation between titers of the HI test and the NP-ELISA was seen by Snyder *et al.* (1985); and de Boer *et al.* (1990). The HI is more sensitive and rapid than the AGID test, but is convoluted due to existence of 16 HA types of IAV (Zhou, *et al.,* 2003).

The ELISA enables rapid serological diagnosis and is suited for influenza A antibody screening, especially in species which harbor several influenza subtypes (de Boer *et al.,* 1990). There were advantages of the ELISA over the AGID. Besides giving an objective interpretation of the test result, the turn around time was shorter (a few hours compared to 20-72 h for the AGID test), the reagent ready to use; no need to pour agar onto plates, the test could be automated , which reduce

labor and time, the data could be easily saved and analyzed using software. The ELISA test was found to be sensitive enough to be performed on pooled serum samples for the detection of antibody against some viral diseases (Knapen *et al.,* 1994) an approach that to reduces considerably the work and cost of testing. The data from this study have also demonstrated the performance of the ELISA on diluted samples, which indicated that the pooling of serum samples could be used with ELISA, not AGID. Furthermore the AGID test may not be suitable assay to test serum from other species of birds e.g. water fowl, because it do not contain good precipitin Abs (Zhou, *et al.,* 2003).

CONCLUSION AND RECOMMENDATIONS

The unclear picture of circulating subtypes of influenza viruses in the Sudan; put the country in a high level of risk of spreading of the disease in it is virulent form. Especially current theories suggest that AI subtype H5 and H7 viruses of high virulence emerge from viruses of low virulence by mutation (Garcia *et al.,* 1996; Perdue *et al.,* 1998). This is supported by phylogenetic studies of H7 subtype viruses, which indicate that HPAI viruses do not constitute a separate phylogenetic lineage or lineages, but appear to arise from non-pathogenic strains (Rohm *et al.,* 1995; Banks *et al.,* 2000). It appears that such mutations occur only after the viruses have moved from their natural wild bird host to poultry. However, the mutation to virulence is unpredictable and may occur very soon after introduction to poultry, or after the LPAI virus has circulated for several months (EFSA, 2005). Further study is needed in order to identify the AIV subtypes that circulate in the Sudan. According to the revised nomenclature for influenza viruses (WHO, 1971) HA and NA should be identified to provide a full description of influenza viruses.

APPENDIX

1. Normal Saline

Normal saline was prepared as illustrated by Webster *et al.,* 2002.

i. Stock 20 time concentrated (20×) normal saline was prepared by dissolving 170 g of NaCl in distilled water for 1000 ml. Sterilized by autoclaving at 121°C.

ii. Physiological saline of 0.85% NaCl was prepared by adding 50 ml 20× stock solution to distilled water. Sterilized by autoclaving at 121°C. Stored at 4 °C for no longer than 3 weeks.

2. Phosphate Buffered Saline-PBS

PBS was prepared as illustrated by Webster, *et al.,* 2002.

i. stock solution 25 time concentrated (25×) Phosphate buffer was prepared by adding to 100ml: 2.74g dibasic sodium phosphate (Na_2HPO_4) and 0.79g monobasic sodium phosphate monohydrate ($NaH_2PO_4 \times H_2O$).

ii. 40 ml of 25× phosphate buffer added to 8.5 g of NaCl and completed to 1 liter by distilled water, mixed thoroughly and pH was checked and adjusted to 7.2 if necessary. Autoclaved and stored at 4 °C for no longer than 3 weeks.

3. Alsever's Solution

The Solution was prepared as described by Hierholzer and Suggs (1969)

i. 1 liter of solution was prepared by dissolving: 20.5 g of glucose, 8.0 g of tri-sodium citrate dehydrate ($Na_3C_6H_5O_7 \times 2H_2O$), 0.55 citric acid ($C_6H_8O_7$) and 4.2 g sodium chloride (NaCl) in distilled water.

ii. pH = 6.1 was checked, and adjusted if necessary.

iii. Sterilized by membrane filtration (membrane filter with 0.22 μm pore size).

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